Supplementary methods

Mutant library

The method of generating a random mutant library and high-throughput screening for TRPV3 mutants is essentially the same as described previously[1]. Error prone PCR (Diversify PCR random mutagenesis kit, Clontech) was performed on the cloned mouse TRPV3 gene and subsequently the PCR product was cloned into the pcDNA5-FRT mammalian expression vector. Previous studies in our lab found that the rate of mutation is approximately 5 to 6 base changes per clone (~4 amino acid substitutions) which led to high occurrence (almost 75%) of inactive mutants[1]. To decrease the rate of mutation, we digested the PCR product using a unique AVRII site, thereby generating two fragments of ~1.6 kb and 0.9 kb size representing the two halves of the gene. We then cloned each fragment into pcDNA5 containing the wild-type TRPV3 gene, from which the corresponding fragment has been cut. Therefore, three mutant libraries including the full-length and N- and C-terminal halves of mouse TRPV3 were generated. Sequence analysis of 10 randomly picked clones confirmed the total number of mutations per clone was ~2 amino acid changes. About 5000 transformants for each library were picked and grown in 96-deep-well plates containing 1.5ml of TB per well. Plasmid DNA was isolated using 96-well plasmid miniprep consumables. DNA concentration was quantified and normalized to 40 ng/μl using a 96-well UV spectrophotometer and an automated pipetting station (MWB Biotech).

Mutagenesis

All site-directed mutants were generated by QuikChange® II XL Site-Directed Mutagenesis Kit (Stratagene). Domain swapping between mTRPV3 and other TRP channels was performed using overlapping PCR and the QuikChange® II XL Site-Directed Mutagenesis Kit, and all clones were verified by DNA sequencing. To generate mutant libraries containing 45 random point-mutations for each amino-acids between Leu642 and Thr660 of mouse TRPV3 and between E638 and P643 of Xenopus TRPV3, we designed primers that randomized the three nucleotides coding each amino-acid (NNN) flanked by nucleotides that correctly encode other amino-acids adjacent to this specific position.

Cell-culture/Transfection

Using a Hewlett Packard Minitrak devise we plated DNA in quadruplicate into ploy-D-lysine-coated 384-well clear-bottom assay plates (Greiner) at 75 ng/well. Positive (wild-type TRPV3) and negative (mock transfection) assay controls were included. Transfections were done by adding Fugene 6.0 (Roche) (0.2 µl/well) diluted in OPTI-MEM (Invitrogen) and trypsinised HEK293T cells (8000 cells/well) to all wells. Transfected HEK293 cells were grown at 37°C, 5% CO2 in Dulbecco's minimal essential medium containing 4.5 mg/ml glucose, 10% heat-inactivated fetal bovine serum, 50 units/ml penicillin, and 50 µg/ml streptomycin. Two days after transfection cells were washed with HANKS buffer by an Embla plate-washer (Molecular Devices), loaded with the calcium-sensitive fluorophore Fluo3 (Molecular Devices) for 1.5 h, washed again and transferred to a FLIPR plate-reader (Molecular Devices) to monitor fluorescence.

Temperature device

The device consists of six thermoelectric modules (HP199-1.4-0.8P, TeTechnology) that are in contact with an aluminium plate with 384 individual pins on the opposite side. The pins fit the wells of a 384-well plate and thus allow the transfer of thermal energy between the metal plate and the liquid in each sample well. Excess thermal energy is dissipated from the opposite side of the thermoelectric modules by a hollow aluminium heat/cold sink in thermal contact with the thermoelectric modules. The temperature of the heat/cold sink is maintained by water circulation from a temperature-stable pump (F-12, Julabo). All thermoelectric modules are controlled by a PID controller (5C7-362, McShane Inc.) and electrically supplied by a power supply (PS-24-20, TeTechnology). Instantaneous temperature values for controller feedback are obtained from a thermistor (TS67-170, McShane Inc.) buried in the metal plate. The PID controller is controlled via an interface with a PC and Mc362 software (McShane Inc.). Actual temperature-readout relevant for measurements is performed optically, through the amplitude of the fluorescent signal of the linearly temperature-sensitive dye[2] $[Ru(bpy)_3]^{2+}$ (Sigma) in multiple sample wells (50 µl at100 µM). The temperature during any given time-point is calculated by extrapolating start- and end-values of a temperature ramp. Ramp-speeds of 2 °C/s with accuracy of ±0.1 °C and plate temperature-homogeneity of < 0.5 °C are achieved.

FLIPR data analysis

Screening data were analyzed with IGORPro (Wavemetrics). Raw data were pooled from all plates. Maximal counts were calculated after individual baseline subtraction. Time-curves for compound-activation were fitted for each single well by a mono-exponential function to obtain rate-values. Histograms of maximal activations, their ratios and rates of signal-increases were produced for all mutants, wild-type TRPV3 and pcDNA controls. Histograms of controls were fitted with Gaussian distributions to obtain averages and standard-deviations, which were used to calculate cut-off values for hit selection. In the

primary screen clones were considered hits that fulfilled all cut-off criteria for three or four wells. For the hit-validation, averages and standard-deviations were calculated from 16 temperature-wells, and each eight compound wells for each individual clone. Cut-off values for hit-confirmation were obtained from calculating average values and standard deviations of pooled data from controls from all plates. For concentration-response curves maximal responses were calculated after baseline subtraction. Averages and standard-deviations were calculated from three wells, responses normalized to unity and fitted by a Hill-equation.

Cloning of Xenopus Tropicalis TRPV3

Early juvenile Xenopus Tropicalis were purchased from XenopusOne (Dexter, Michigan). Total RNA was extracted from whole brain of a frog using TRIZOL following the manufacturer's protocol (Invitrogen, CA, USA). First strand cDNA was prepared from the total RNA using SuperScript II reverse transcriptase (Invitrogen, CA, USA). PCR was done using a pair of primers, sense:

5' -ATGTTAACTGATCTCCTTTCAGAAGCAAAGTCCAGTTCAAGGG - 3' and antisense: 5' - TTACATACCAGGAAAAAATGTTAACCCAGGC- 3' were designed to amplify putative full-length *Xenopus tropicalis* TRPV3 sequences (http://genome.jgi-psf.org/Xentr3/Xentr3.home.html). The PCR product was cloned into a pCR2.1-TOPO vector ((Invitrogen, CA, USA) and subcloned into pcDNA5-FRT vector using Kpn I / Not I sites. Transient transfection of *Xenopus tropicalis* TRPV3 into HEK293 cells failed to give any responses to 2-APB, camphor and heat (from 25 to 42 C°) probably due to missing N- and C-terminal sequences (predicted xTRPV3 is considerably shorter than mammalian TRPV3s). To obtain a functional *Xenopus* TRPV3 channel we added 134 amino acids (position 1 to 134) of mouse TRPV3 to the N-terminal of the *Xenopus* TRPV3 right downstream of the predicted methionine by overlap PCR. Similarly, 37

amino-acids of mouse TRPV3 C-terminal (position 755 to 791) were added to the end of *Xenopus* TRPV3 (with deletion of the last 7 predicted amino acids of *Xenopus* TRPV3).

Electrophysiology

HEK293 cells were grown in Dulbecco's minimal essential medium (DMEM) containing 4.5 mg/ml glucose, 10% heat-inactivated fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. For patch clamp recording, cells were co-transfected with EGFP and wild-type or mutant mTRPV3 constructs in wells of 24-well plates using Fugene 6.0 (Roche) following the protocol provided by the manufacturer. Transfected HEK293 cells were reseeded on 12 mm round glass coverslips (Warner Instruments) one day after transfection. Whole cell recordings were performed the following day. Recording pipettes were pulled from micropipette glass (Sutter) to 2–4 MΩ when filled with a pipette solution containing 140mM CsCl, 0.6 mM $MgCl_2$, 10 mM BAPTA, 10 mM Hepes, pH 7.20, and placed in the bath solution containing 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM Hepes, pH 7.4. Isolated cells were voltage-clamped in the whole cell mode using an EPC9 (HEKA Instruments Inc) amplifier. Voltage commands were made from the Pulse and PulseFit program, and the currents were recorded at 5 kHz. Voltage ramps of 100 ms to +100 mV after a brief (20 ms) step to -100 mV from holding potential of 0 mV were applied every second. Cells were continuously perfused with the bath solution through a Valve-Bank perfusion system (Automate Scientific). For experiments addressing the temperature activation of TRPV3 in HEK293 cells, the solution was heated using a CL-100 temperature controller (Warner Instruments) and an SC-20 Solution In-Line Heater/Cooler (Harvard Apparatus). Temperature was recorded with a thermistor placed <0.5 mm from the cell. Stock solutions of 2APB, camphor were made in DMSO. Whole cell experiments were performed at 25°C. For inside-out patches, both the pipette solution and bath solution contained (in mM) 140 CsCl, 1 EGTA, 1 MgCl2, 10 Hepes, pH 7.4. The excised patches

were held constantly at desired potentials while 2APB was repetitively applied and washed away from the bath through perfusion. Single channel currents were recorded at 10 kHz and filtered at 3 kHz.

Molecular dynamics simulation

The mouse and xenopus TRPV3 homology models were built using Prime 1.6 software from FirstDiscovery suite[3] and Kv1.2 (2A79.pdb) as a template. The TRPV3 single-point mutants and the chimeric construct hV2mV3-II were prepared by changing corresponding amino-acid residues in the TRPV3 wild-type homology model.

The structures were solvated in TIP3P truncated octahedron box, and periodic boundary conditions were applied. The solvated complexes were subject to minimization, followed by 20 ps of constant volume and subsequent 100 ps of constant pressure MD equilibration runs at 300 K. After the equilibration, 10 ns production runs were performed with constant pressure dynamics at 300K. During the equilibration and production MD the positions of resides other than those in loops were constrained with a harmonic force of 100 kcal/mol/Å^2. Bonds involving hydrogen atoms were constrained via the SHAKE algorithm. A 12 Å cutoff distance was used for all nonbonded interactions. The time step for integration was 2 fs, and the coordinates of all atoms were saved every 5 ps. All simulations were performed using the AMBER 9 software package[4] with the Wang *et al.* force field[5]. All calculations were performed on a cluster consisting of 256 2.4 MHz Opteron's processors. Each MD simulation was run in parallel distributed over 8 processors. It took on average 3 weeks to complete one 10-ns MD simulation run. In order to locate clusters of similar structures along the trajectory we used the K-mean clustering method implemented in the MMTSB toolset[6]. We performed clustering using only loop residues and a clustering radius of 1.5 A. The centres of each cluster were used to represent the range of dynamics in each structure.

References

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Supplementary Figure 1: Histograms of primary screen

a-c, Histograms of collected data for maximal responses to 2APB (a), camphor (b) and temperature (c) for all clones (grey), TRPV3 (red) and pcDNA (blue).

d-g, Histograms of collected data for rates of signal increase for 2APB (d), camphor (e) and ratios of maximal responses for 2APB/temperature (f) and camphor/temperature (g). Black lines are Gaussian fits to histograms.

h, plot of maximal 2APB vs. maximal temperature signal for 44 individual validated hits. Error bars indicate s.d.

Supplementary Figure 2: Histograms of validation screen

a-c, Histograms of collected data of the validation screen for maximal responses to temperature (a), 2APB (b) and camphor (c) for all selected clones (grey), TRPV3 (red) and pcDNA (blue). Black lines are Gaussian fits to histograms.

Supplementary Figure 3: Dose-response and heat-activation curves for single-point mutants

a, Normalized dose-responses for single-point mutants Met440Val, Phe445Ile, Tyr451Asn, Asn452Ile, Ile453Asn andTyr461Phe (all red) and TRPV3 wild-type (black) for 2APB (squares) and camphor (circles). Lines are fits of Hill-equations to the data. Error bars indicate s.d., n=3. Note that mutant Tyr451Asn appears

wild-type-like, but was discarded because of a significantly right-shifted camphor dose-response, compared to wild-type TRPV3.

b, Background-subtracted average responses of respective mutants and TRPV3 wild-type upon heat-stimulation. Error bars indicate s.d., n=16.

Supplementary Figure 4: Unitary conductance of Ile644Ser, Asn647Tyr, Tyr661Cys and wild-type TRPV3.

a, 2APB-dependent TRPV3 wild-type single-channel current recordings from inside-out patches during a voltage-step protocol.

b, example of single-channel current-amplitude histogram (TRPV3 wild-type at +120 mV). Red curves are Gaussian fits and were used to obtain current differences and standard deviations.

c, single-channel current-amplitudes obtained from Gaussian fits to current-histograms as a function of voltage. Error bars are s.d., straight lines linear fits to the data. Average values±s.d. of unitary conductance are 112±4 pS (Ile644Ser), 109±6 pS (Asn647Tyr), 96±6 pS (Tyr661Cys) and 101±5 pS (wild-type TRPV3).

Supplementary Figure 5: FLIPR responses to compounds and temperature for 18 amino-acid changes at position Ile644.

a, Normalized dose-responses for 18 single-point mutants (all red) and TRPV3 wild-type (black) for 2APB (squares) and camphor (circles). Lines are fits of Hill-equations to the data. Error bars indicate s.d., n=3.

b, Average response of each mutant to 30 μM 2APB without prior heat-stimulation. Error bars indicate s.d., n=16.

c, Background-subtracted average response of each mutant and TRPV3 wild-type (Ile) upon heat-stimulation. Error-bars indicate s.d., n=16.

Supplementary Figure 6: FLIPR responses to compounds and temperature for 18 amino-acid changes at position Asn647.

a, Normalized dose-responses for 18 single-point mutants (all red) and TRPV3 wild-type (black) for 2APB (squares) and camphor (circles). Lines are fits of Hill-equations to the data. Error bars indicate s.d., n=3.

b, Average response of each mutant to 30 μM 2APB without prior heat-stimulation. Error bars indicate s.d., n=16. Asn647Ser had wild-type-like 2APB response (data not shown).

c, Background-subtracted average response of each mutant and TRPV3 wild-type (Asn) upon heat-stimulation. Error-bars indicate s.d., n=16. Asn647Ser had wild-type-like heat response (data not shown).

Supplementary Figure 7: Detailed screen in mouse TRPV3

Fractions of phenotypes observed in single point-mutation screen for mouse TRPV3 resulting from responses to heat, 30 μM 2APB and 1.75 mM camphor. The total number of tested clones was 45 for each residue.

Supplementary Figure 8: Detailed screen in Xenopus TRPV3

a, Average FLIPR responses of xenopus TRPV3 (red) and pcDNA (blue) upon heat-stimulation (below), n=64.

b, Normalized FLIPR dose-response for xenopus TRPV3 for 2APB. Lines are fits of Hill-equations to the data. Error bars indicate s.d., n=2.

c, Fractions of phenotypes observed in single point-mutation screen for xenopus TRPV3, resulting from responses to heat and 30 μM 2APB. The total number of tested clones was 45 for each residue.

Supplementary Figure 9: Chimeric constructs

a, Alignment of amino-acid sequences of TRPV channels used for constructing chimeric channels. Red bars indicate positions of domains (I-V) that were exchanged for different constructs. Abbreviations are h: human, m: mouse, r: rat, x: xenopus. Colours in the sequence of TRPV3 indicate locations of TM5 (blue), pore region (green), selectivity filter (orange), TM6 (pink) and identified point-mutations (yellow).

b, Structural models of one subunit with colours corresponding to the alignment.

Supplementary Table 1: criteria for hit selection

Values for TRPV3 wild-type and pcDNA5 indicate averages±s.d (n=580), obtained from Gaussian fits to the respective distributions (Supplementary Fig. 1). Cut-off values for mutant clones were derived from these averages and multiples of their standard deviations.

Criteria	**TRPV3 wild-type**	**pcDNA5**	**Cut-off values for clones**
temperature	-	2159±550	$x < 2710$
2APB	36339±16902	-	$x > 19437$
camphor	8926±3486	-	$x > 5441$
2APB-rate / s^{-1}	6.8±1.6	-	$5.2 < x < 11.6$
camphor-rate / s^{-1}	39.1±20.2	-	$19.0 < x < 99.6$
2APB/temperature	4.5±2.1	-	$x > 10.6$
camphor/temperature	1.1±0.8	-	$x > 3.5$

Supplementary Table2: criteria for hit validation

Values for TRPV3 wild-type and pcDNA5 indicate averages±s.d. (n=240) obtained from Gaussian fits to the respective distributions (Supplementary Figure 2). Cut-off values for mutant clones were derived from these averages and multiples of their standard deviations.

Criteria	TRPV3 wild-type	pcDNA5	Cut-off values for clones
temperature	-	1454±332	x < 2118
2APB	31616±6125	-	x > 19366
camphor	25455±6433	-	x > 12590

Supplementary Table 3: mutations and EC_{50} values of confirmed hits

Single-point mutations of 15 confirmed hits and their respective EC_{50}-values for activation by 2APB and camphor. Mutations that were individually engineered are shown in bold. Errors are s.d., n=15 for wild-type TRPV3 and n=3 for mutants.

Mutations	**EC_{50} (2APB) / μM**	**EC_{50} (camphor) / mM**
TRPV3 wild-type	8.7±2.7	1.4±0.2
Thr17Ser, Ala302Val, **Tyr461Phe**	5.3	1.1
Asn79His, Ala245Val, **Met440Val**	12.4	1.6
Asn647Tyr	13.4	1.4
Ile595Phe, **Leu657Ile**	6.8	1.0
Ile289Thr, **Lys434Met**, **Leu670Phe**, Phe780Leu	14.0	1.9
Ala32Glu, Ser77Pro, Ile204Asn, **Thr660Ser**, Met717Val	12.3	1.8
Tyr409His, **Asn643Ser**, Leu720Pro	10.8	1.5
Ile186Glu, Asn220Ser, **Tyr461Phe**, Phe489Ser	6.1	1.4
Ile123Phe, **Ile453Asn**, Ile765Thr	11.0	1.4
Gln114Arg, Ile289Val, Thr397Ser	11.3	1.6
Lys358Glu	10.6	1.3
Asn71Thr, Asn197Ser, Lys774Asn	8.3	1.3
Ile644Ser	5.1	1.1
Tyr451Asn, **Tyr661Cys**	5.6	1.3
Phe445Ile, Trp481Arg	6.6	1.6

Supplementary Table 4: additional point mutations in mouse TRPV3

Sequencing results for single-point mutations that specifically affect heat-activation in mouse TRPV3.

Position	Mutation
Ser648	Ile
Pro651	Ala
Phe654	Ser, Lys
Leu657	Glu

Supplementary Table 5: point mutations in xenopus TRPV3

Sequencing results for single-point mutations that specifically affect heat-activation in xenopus TRPV3.

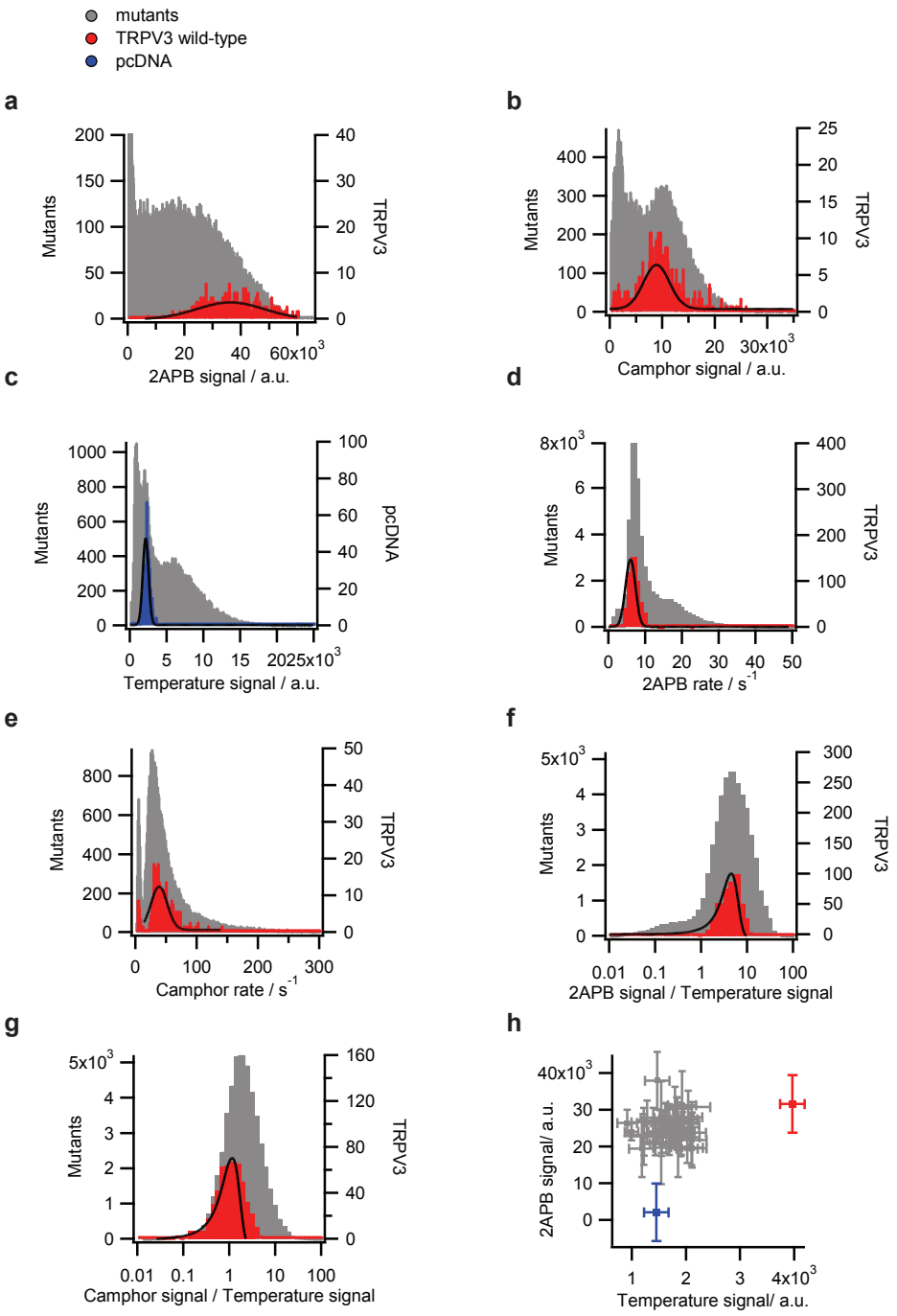
Position	Mutation
Glu638	His, Lys, Phe
Met639	Ala, Arg, Glu, Gly, Lys, Ser, Thr, Trp, Val
Asp640	Leu, Lys, Tyr, Val
Lys641	Pro
Asp642	Met, Pro, Tyr
Pro643	Leu

Supplementary Table 6: chimeras

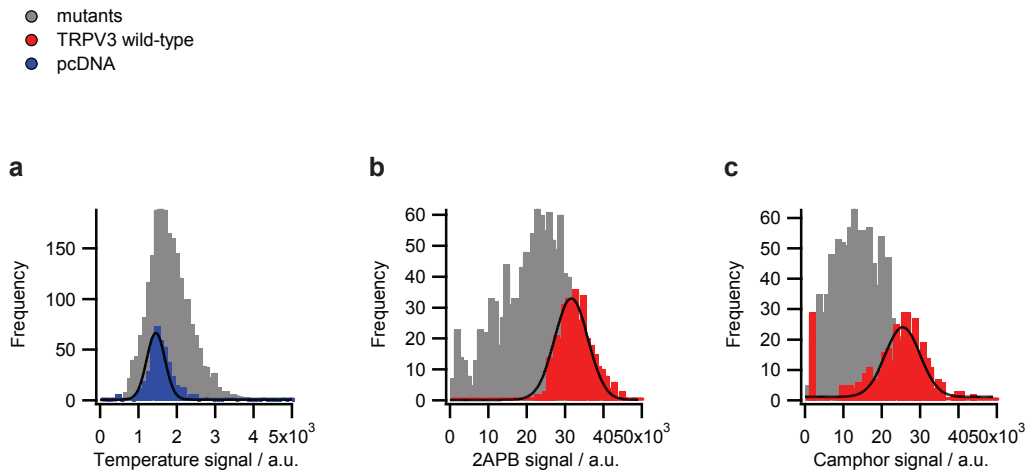
Chimeras and their tested responses to stimulation by compounds (2APB, camphor, THC or capsaicin) and heat. Chimeras are ordered for constructs that were aimed to investigate necessity (1) or sufficiency (2) of the replaced domain and named with the insert first and the background second. Species are abbreviated h: human, m: mouse, r: rat, x: xenopus. Numbers indicate inserted fragment as illustrated in Supplementary Figure 9.

Chimera		Compound tested	Compound response	Heat response
1.	rV1mV3-II	2APB, camphor	EC50 shifted	reduced
	hV2mV3-I	2APB, camphor	EC50 shifted	no
	hV2mV3-II	2APB, camphor	like mV3 wild-type	no
	rV2mV3-II	2APB, camphor, THC	EC50 shifted	no
	rV4mV3-II	2APB, camphor	EC50 shifted	no
	mV6mV3-I	2APB, camphor	no response	no
	mV6mV3-II	2APB, camphor	no response	no
	mV6mV3-III	2APB, camphor	no response	no
	mV6mV3-IV	2APB, camphor	no response	no
	mC3mV3-II	2APB, camphor	no response	no
	xV3mV3-II	2APB, camphor	no response	no
2.	mV3hV2-I	2APB, THC	no response	no
	mV3hV2-II	2APB, THC	no response	no
	mV3hV2-V	2APB, camphor	no response	no

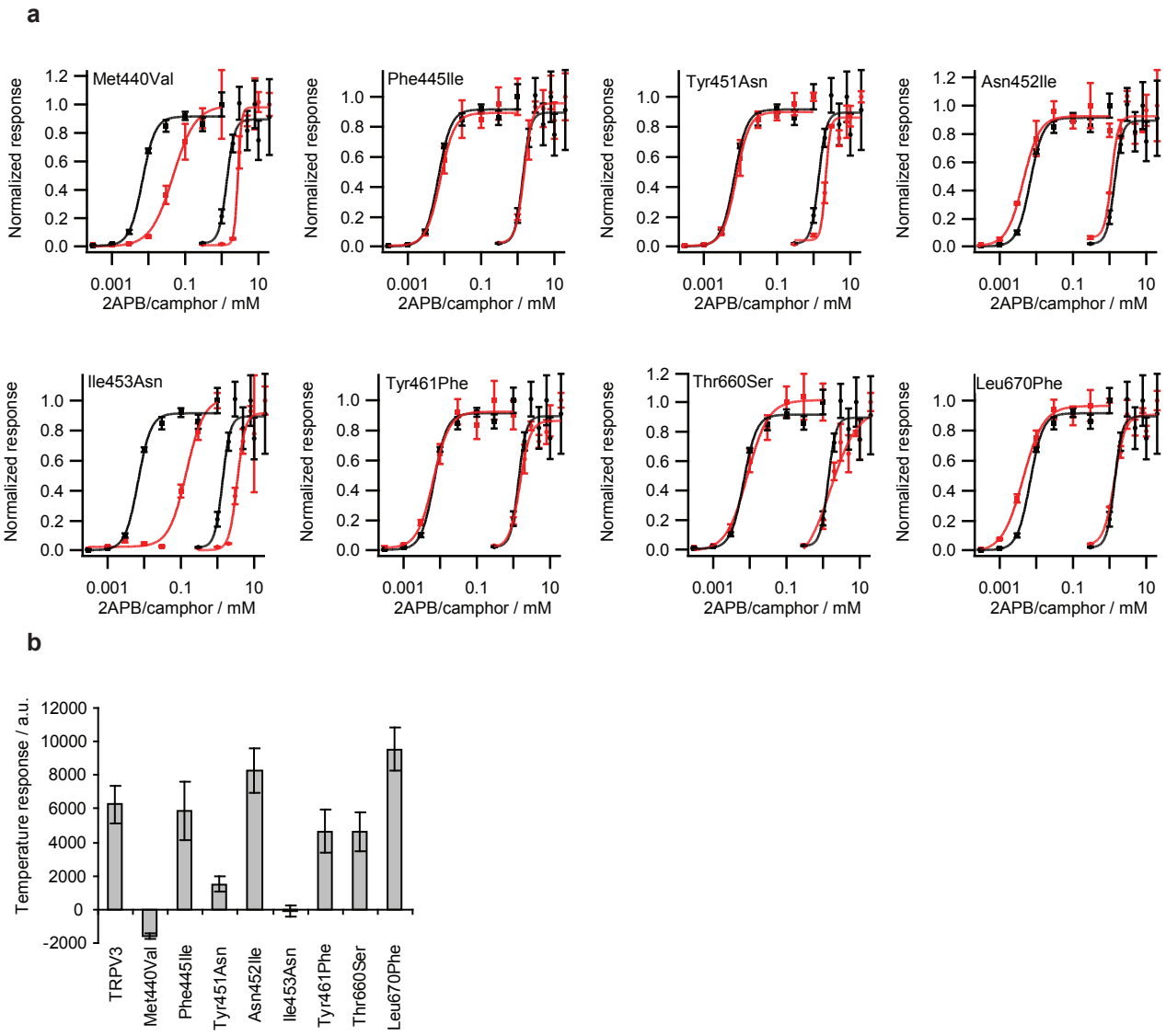
Supplementary Figure 1



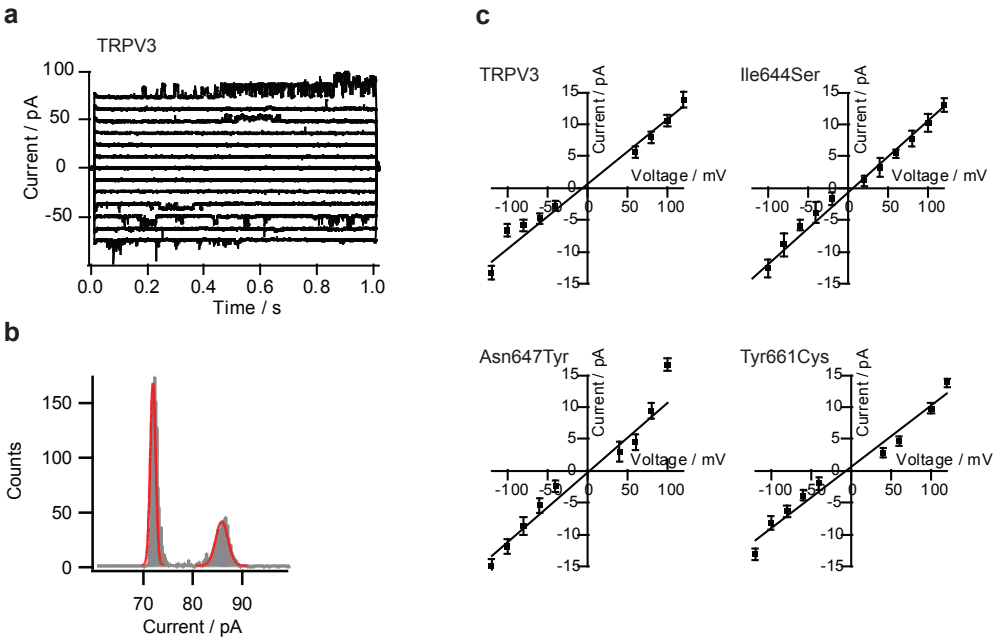
Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4



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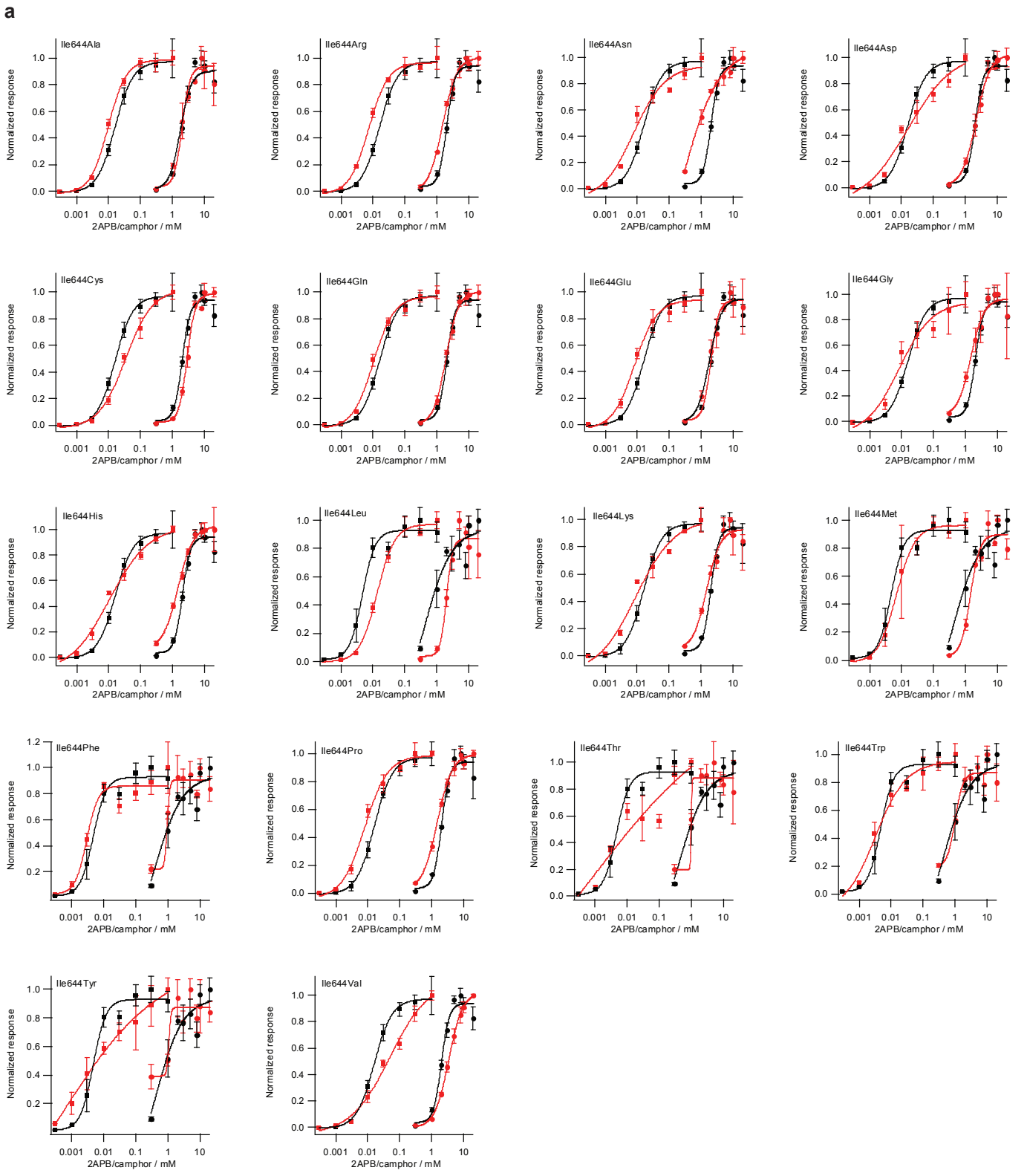

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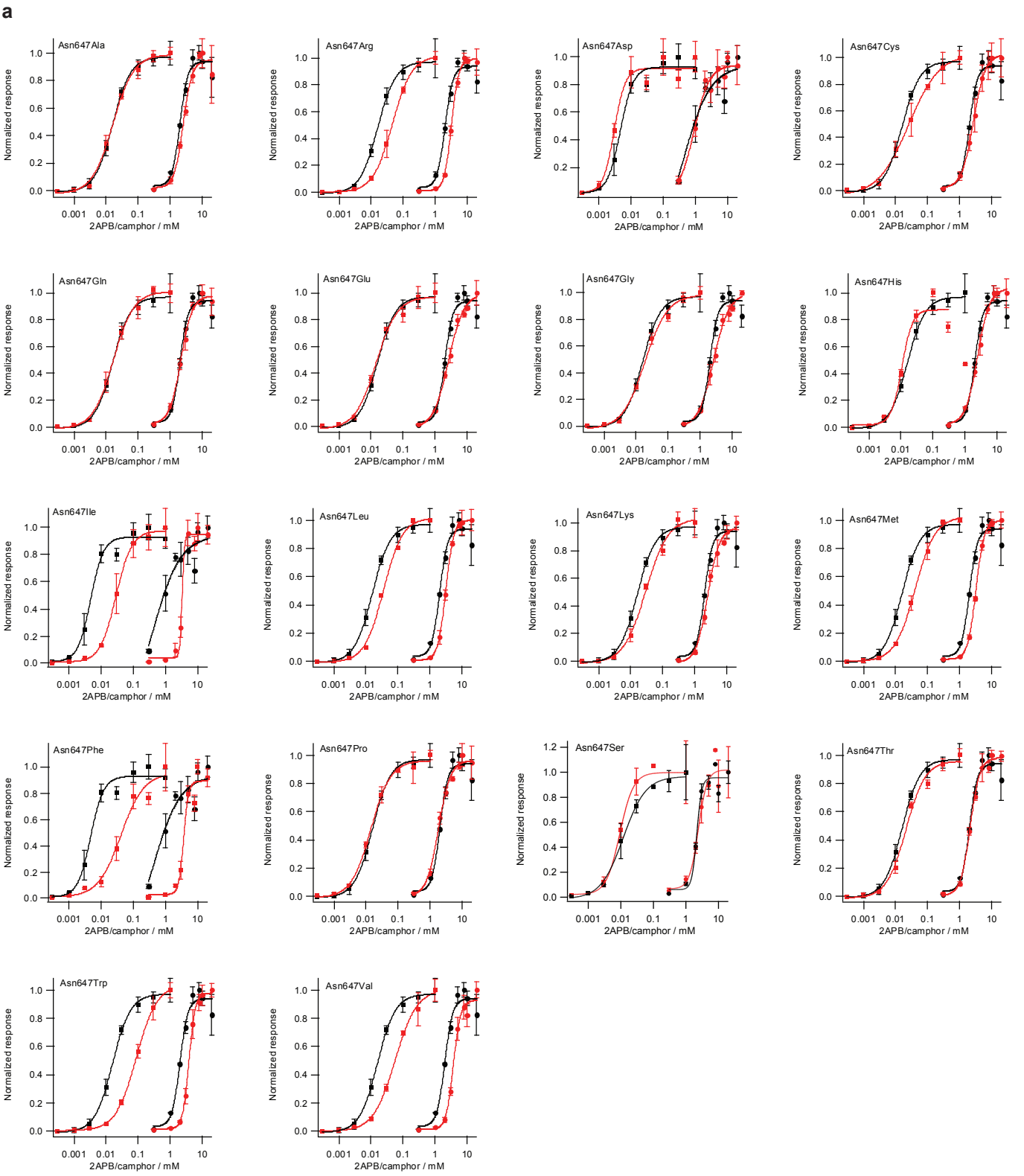
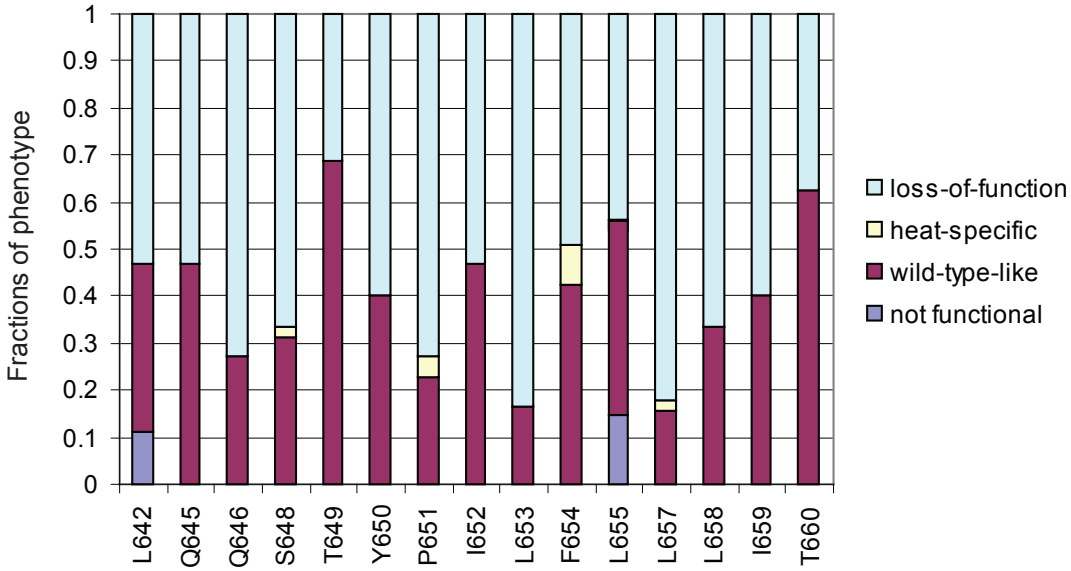
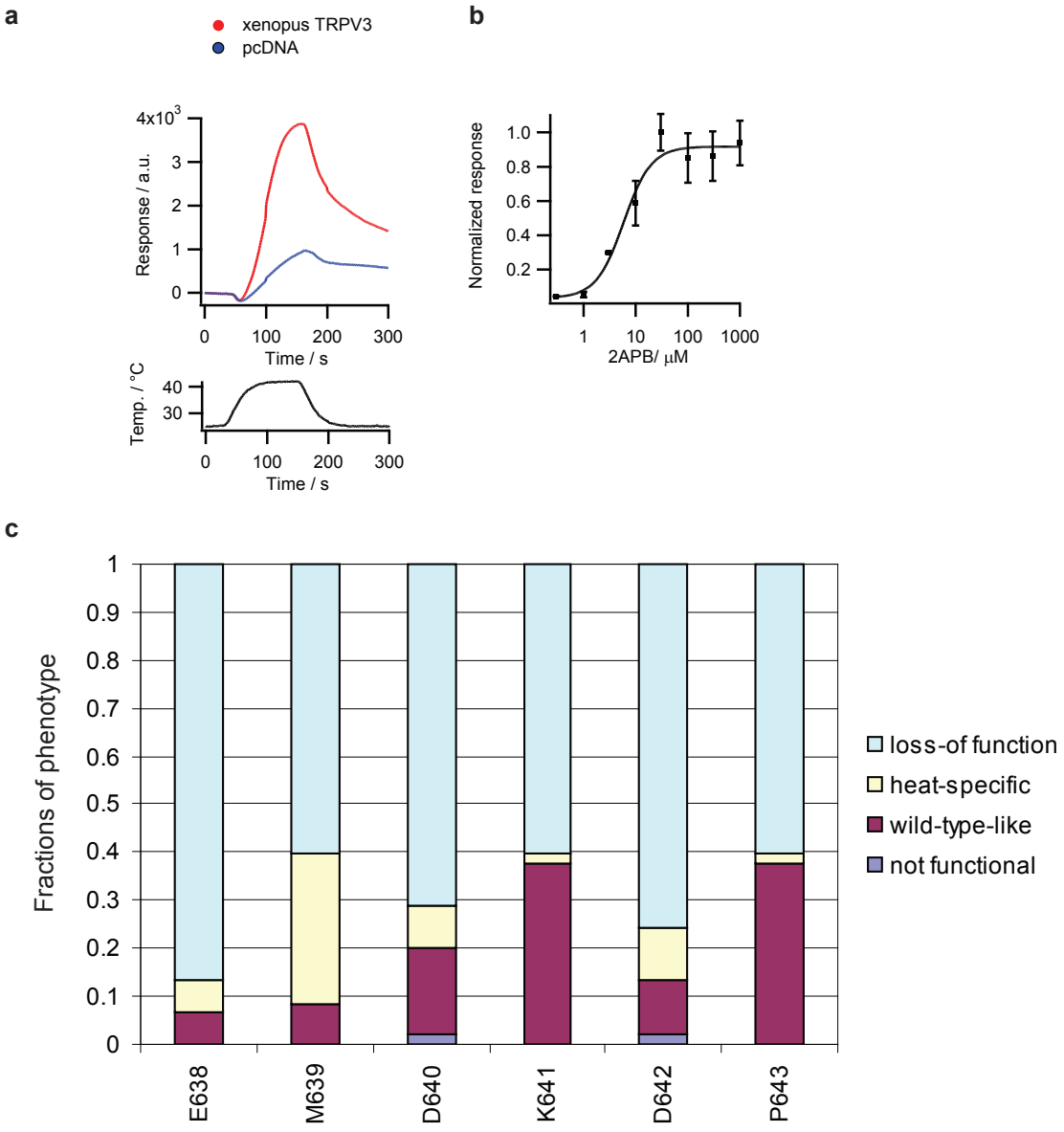


Supplementary Figure 7

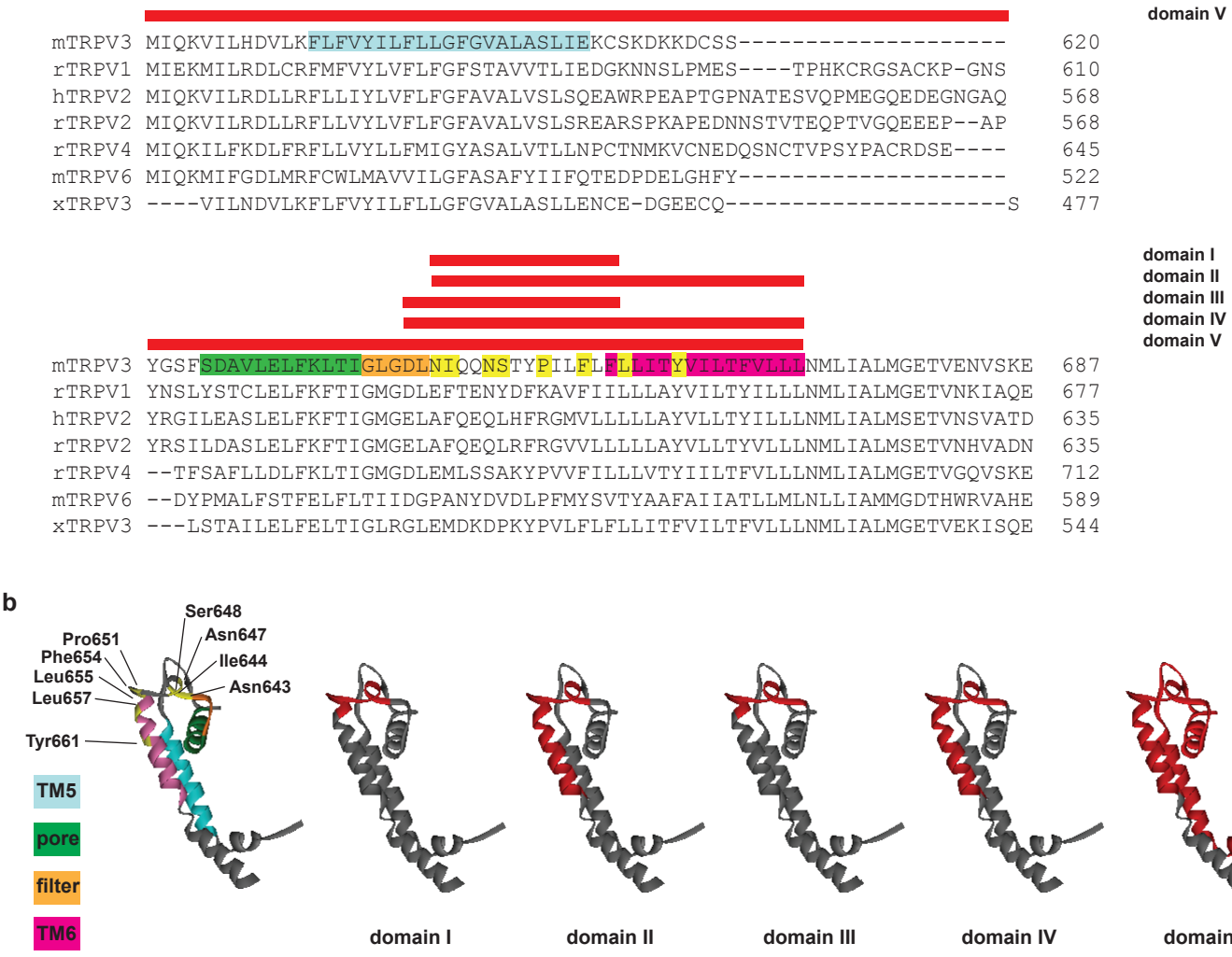


Supplementary Figure 8



Supplementary Figure 9

a



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