Supplementary Information for
The Acquisition of Cold Sensitivity during TRPM8 Ion Channel Evolution

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- Supplementary text
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- Legends for Datasets S1 to S2
- SI References

Other supplementary materials for this manuscript include the following:
- Datasets S1 to S2
Supplementary Information Text
Materials and Methods
Phylogeny and Positive selection analysis
The group of homologous *trpm8* genes were aligned by MUSCLE using MEGA X (1), all gaps (“-”) and unknown sites (“N”) were removed before positive selection tests. The phylogeny tree was constructed by TimeTree (2) and used as an input tree in the PAML test. The CODEML program in the PAML package was employed to analyze positive selection genes (3). The dN/dS ratios (\(\omega\)) of the *Xenopus tropicalis* or mammal branch were estimated with the branch-site model of PAML. The log-likelihood of the positive detection model (InL1: Model A Alternative hypothesis, fix_omega = 0) and the corresponding null model (InL0: Model A Null hypothesis, fix_omega = 1) were calculated. 2ΔInL is the double time of absolute of InL1 minus InL0. P value is the right tail probability of the \(\chi^2\) distribution of 2ΔInL. Positively selected gene (PSG) were inferred if P value < 0.01. After identifying PSG, the Bayes empirical Bayes (BEB) method was utilized to calculate posterior probabilities and positively selected sites.

Skeletal staining
The tissues from fishes and frogs were fixed and dehydrated with 10% formalin for one week. The fixed tissues were washed in distilled H\(_2\)O and bleached with 0.1% H\(_2\)O\(_2\) for one or two days. The samples were stained with 0.1% Alcian blue (pH 2.5) for 4 days followed by washing with distilled water. The specimens were then incubated in 0.5% KOH saturating Alizarin red solution for 5 days, then dehydrated briefly in xylene. The stained tissues were stored and photographed at 100 % glycerol.

cDNA and plasmid construction
The cDNA sequences of TRPM8 orthologs with their corresponding NCBI code are: *Rhinatrema bivittatum trpm8* (115094677), *Xenopus tropicalis trpm8* (100302008), *Chelonia mydas trpm8* (102947845), *Gopherus evgoodei trpm8* (115659981), *Gavialis gangeticus trpm8* (109301952), *Python bivittatus trpm8* (103065492), *Pogona vitticeps trpm8* (110086114) and *Camelus bactrianus trpm8* (105063570). These cDNAs were synthesized by Tsingke (Beijing, China) and subcloned into the pCDNA3.1 vector.
All single-point mutants and chimeras were constructed using Fast Mutagenesis Kit V2 (SBS Genetech) following the manufacturer's instruction. Primers designed to generate chimeras and mutations are listed in SI Appendix, Datasets S1 and S2. All channel mutants and chimeras were confirmed by DNA sequencing.

Cell culture and transient transfection
HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C with 5% CO\(_2\). The transient transfections with TRPM8 cDNA vectors were performed using the Lipofectamine 2000 reagent (Invitrogen) following the instruction manual. After transfection for 24 hours, the responses of cells to menthol and temperature were tested by electrophysiological recordings.

Electrophysiology
Patch-clamp recordings were performed by using an EPC10 amplifier (HEKA) controlled by PatchMaster software (HEKA). Patch pipettes were made from borosilicate glass and fire-polished to a resistance of ~3 M\(\Omega\). Both the pipette solution and the bathing solution contained 130 mM NaCl, 0.2 mM EDTA and 3 mM HEPES (pH 7.2). The membrane potential was held at 0 mV. The whole-cell currents were recorded from a step to +80 mV for 200 ms followed by another 200 ms step to -80 mV. The current signals were filtered at 2.9 kHz and sampled at 10 kHz. The perfusion and solution switching were carried out by a gravity-driven system (RSC-200, BioLogic). The solutions were flowed through separated tubes to minimize mixing of the solutions. The patches were placed at the perfusion tube outlet. To record the ANAP-incorporated channels, the emission spectrum of ANAP was imaged and analyzed as described below.

Temperature control
To record the cold activation currents, cells were first placed in preheated bath solutions (32°C), which prevented the cold activation of TRPM8 channels. Patched cells were placed at the perfusion tube outlet for further cooling assays. Precooled bath solution was prepared by embedding solution reservoirs in ice water and flowed through a perfusion tube to activate the channels. A TA-29 miniature bead thermistor (Harvard Apparatus) was placed right next to the patched cell and monitored the local temperature accurately. The digital readout of thermistor was fed into an analog input of the patch clamp amplifier and recorded simultaneously with electrophysiological assays.

**Fluorescence unnatural amino acid**

L-ANAP methyl ester was purchased from AsisChem. pANAP vector was purchased from Addgene. ANAP was incorporated into TRPM8 protein by introducing a TAG amber stop codon mutation. Transfection steps were as follows: 3 μg channel expression plasmid and 1 μg pANAP vector were co-transfected into HEK293 cells; after the transient transfection, transfection solution was removed and replaced by fresh medium containing 20 μM L-ANAP for 24 hours; ANAP containing culture medium was replaced by fresh medium for at least 4 hours before electrophysiological tests or spectrum analysis.

ANAP fluorescence was excited by the X-Cite XYLIS light source with a 340-390 nm excitation filter and a 420LP emission filter (>420 nm) on an inverted fluorescence microscope (Olympus IX73) using a ×20 objective. The emission spectrum of ANAP was imaged with an Acton ARC-SP 2156 Imaging Spectrograph (Princeton) in conjunction with an Orca R2 C10600-10B CCD camera (Hamamatsu). The ANAP emission peak value was measured by fitting the recorded emission spectrum with a skewed Gauss distribution in Igor Pro version 6.37.

**Calculation of ΔH and ΔS**

The changes in enthalpic (ΔH) and entropic (ΔS) induced by cold-driven transition were calculated by constructing Van’t Hoff plots and fitting them to the equation \( \ln K_{eq} = -\Delta H/RT + \Delta S/R \), where \( K_{eq} \) represents the equilibrium constant measured from the cold-driven TRPM8 open probability, \( K_{eq} = P_o/(1-P_o) \), in which \( R \) represents the gas constant, and \( T \) represents temperature in Kelvin. The open probability induced by cold was determined as the ratio between the macroscopic current (after correcting for temperature-dependent single-channel conductance) and the estimated maximum current using noise analysis (4).

**Molecular modeling**

To model *Xenopus tropicalis* TRPM8 in the apo state, loop modeling was performed using the Rosetta molecular modeling suite version 2020.27 (5). The cryo-EM structure of TRPM8 (PDB ID: 6O6A) was selected to be the homologous template (6). The nonhomologous regions were modeled de novo by the Kinematic closure (KIC) loop modeling protocol (7, 8). In each round, modeling was set to generate 10000 models. The energy scores of these models were ranked. The top ten models were used as input for the next round of modeling. After several rounds of KIC loop modeling, the top ten models converged well. The model with the lowest energy was finally selected as the closed state model. This model was further refined by the relax application within the Rosetta suite (9).

To obtain the cold-induced open state, the *Xenopus tropicalis* TRPM8 was modeled by a similar procedure. After each round of KIC loop modeling, the output models were filtered. The changes of solvent accessible surface area (SASA) values measured from ANAP imaging experiments were set as filtered factors. From the experiment, we observed an increase in ANAP emission peak at 166, 360, 453, 537, 959 and 960 sites. The shifts of ANAP emission peak indicate that the side chain of these amino acids transit from hydrophobic to hydrophilic environment (10), which is accompanied by an increase in SASA of the sidechain (11). In contrast, a decrease in ANAP emission peak at 413 site was detected. This indicates the side chain transit from hydrophilic to hydrophobic environment, which is accompanied by a decrease in SASA of the side chain. Among filtered models, the top ten models ranked by energy were selected and input for the next round of loop modeling. After several rounds of KIC loop modeling, the top ten models...
converged well. The model with the lowest energy was finally selected as the open state model and further refined by the relax application within the Rosetta suite (9).

SASA of each amino acid residue in models was measured by RosettaScripts within the Rosetta suite (12). Command lines employed in Rosetta to perform the modeling processes, the SASA measurements, and filtering are appended. Pore radius of XtTRPM8 models was calculated by the HOLE program version 2.0 (13, 14). All the molecular graphics of TRPM8 models were rendered by UCSF Chimera software version 1.12 (15).

**Preparation of recombinant MHR1-3 domain**
The optimized plasmid encoding species-specific MHR1-3 domain was synthesized and subcloned into expression vector PET43.1A. The encoding plasmid was transformed into the E. coli strain BL21(DE3), the positive clone was selected by ampicillin. Bacteria were cultured in LB medium at 37°C 180rpm. When the OD600 of bacteria increased to 0.6, the expression of MHR1-3 was induced by 0.1 mM IPTG. BL21 was further cultivated at 28°C 100 rpm for 4 hours. The thalli were collected by centrifugation at 8000 rpm for 3 min and resuspended in precooled NTA buffer with 0.1 mg/ml lysozyme. The expression protein was extracted from bacteria by cell ultrasonication and captured by His6 tag affinity purification. The renaturation of inclusion body protein was carried out by gradient dialysis. The target protein was lyophilized and further purified by a fast protein liquid chromatography (pure 25M, AKTA).

**Gel filtration chromatography**
A 140 ml glass column with an internal diameter of 1.6 cm was packed with 112 ml Sephacryl S-100 HR resin. The column was equilibrated with PBS buffered saline (20 mM Na2HPO4, NaH2PO4, pH 6.0, 15 ml/h). The samples were eluted with the same buffer by using a fast protein liquid chromatography (pure 25M, AKTA). The absorbance of the collected fractions was recorded at 215 nm using UV monitor U9-M.

**Circular dichroism spectroscopy**
Protein samples were dissolved into Phosphate buffer (10 mM NaH2PO4, pH7.2, 0.5 mg/ml). Circular dichroism (CD) spectra of proteins were executed by a Jasco J-810 spectrophotometer under constant N2 flush. The CD spectra of purified TRPM8 MHR1-3 domain were obtained from 190 nm to 250 nm at cold (10°C) or moderate (30°C) temperature with 10 mm pathlength circular cuvettes. The baseline was corrected by the protein storage buffer. All data were collected using a step resolution of 1 nm, a scan speed of 50 nm/min, and a response time of 1 s. CD spectra values were converted to units of molar ellipticity (θ).

**Commands in Rosetta to perform loop modeling**
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   -in:file:fullatom \ 
   -ignore_unrecognized_res \ 
   -in:file:s /###.pdb \ 
   -loops:loop_file /###.loop \ 
   -loops:frag_sizes 9 3 1 \ 
   -loops:remodel perturb_kic \ 
   -loops:refine refine_kic \ 
   -loops:relax no \ 
   -loops:build_attempts 20 \ 
   -relax:bb_move false \ 
   -max_inner_cycles 30 \ 
   -nstruct 10000 \ 
   -out:file:silent /###.silent \ 
   -out:file:silent_struct_type binary \ 
   -overwrite
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**Commands in Rosetta to perform SASA calculation and filtering**
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-p:parser:protocol /###.xml 
-ignore_unrecognized_res 
-in:file:silent_struct_type binary 
-in:file:silent /###.silent 
-nstruct 1 
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-overwrite 

Rosetta scripts to perform SASA calculation and filtering

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Statistical Analysis
Experimental data from electrophysiological recordings were analyzed using Igor Pro
(WaveMatrix, version 6.37) and Prism (GraphPad version 8.0.1). All values are given as average ± SEM for the number of measurements indicated (n). Statistical significance was determined using the Student’s t test. Statistical significance was accepted at a level of p<0.01. N.S. indicates no significance.
EC₅₀ values were obtained from fitting a Hill equation to the menthol-induced concentration-response relationship.

\[
\frac{I_x}{I_{max}} = 1 - \frac{[X]^n}{EC_{50}^n + [X]^n}
\]

Where \(I_x\) represents the difference between the steady-state TRPM8 current and the leaking current in the presence of concentration \([x]\). \(I_{max}\) represents the difference between the maximal current amplitude and the leaking current. \(EC_{50}\) is the concentration for the half-maximal effect of menthol activation.

The open probability \((P_0)\) was calculated from the noise analysis equation (4).

\[
N = \frac{I^2}{i * I - \sigma^2}
\]

Where \(N\) is the number of channels in the patched membrane, \(I\) is the ensemble average current measured in the experiment, \(i\) is the TRPM8 single-channel current, and \(\sigma^2\) is the squared
deviations in current amplitude from the average value. The maximum current when all ion
channels were at the open state is equal to \( i \times N \). The open probability was obtained according to
the relationship \( P_0 = I / (i \times N) \).

\( Q_{10} \) was calculated as:

\[
Q_{10} = \left( \frac{I_2}{I_1} \right)^{10/(T_2-T_1)}
\]

Where \( I_1 \) and \( I_2 \) respectively represent the difference between the steady-state TRPM8 current
and the leaking current at temperatures \( T_1 \) and \( T_2 \).
Fig. S1. Sequence alignment of *trpm2*, *trpm8*, and *trpm2*-like gene.

(A) VISTA sequence conservation plot of *trpm8* and *trpm2*-like coding sequences in *Protopterus annectens*. The *trpm2* coding sequence was used as a reference. (B) The exons of *trpm2*, *trpm8*, and *trpm2*-like with more than 50% sequence similarity were aligned using the MUSCLE algorithm and MEGA X program. The sequences with identical nucleotide are highlighted in black.
Fig. S2. Menthol and cold sensitivity of TRPM8 and channel mutants.

(A) Concentration-response relationship of TRPM8 orthologs in the presence of menthol (n = 3). Pv, Pogona vitticeps; Pb, Python bivittatus; Gg, Gavialis gangeticus; Ge, Gopherus evgoodei; Xt, Xenopus tropicalis; Cm, Chelonia mydas; Rb, Rhinatrema bivittatum; Pa, Protopterus annectens.

(B) Van’t Hoff plots for the cold-activated TRPM8 currents shown in Figure 1C. Dotted lines represent fits of the Van’t Hoff equation. ΔH and ΔS were calculated directly from the slope and intercept of the plots. (C) Measured ΔH (filled bars, left axis) and ΔS values (open bars, right axis) of TRPM8 orthologs (average ± SEM; n = 3; N.S., no significance).

(D) Representative current traces of RbTRPM8 (Rhinatrema bivittatum) and the channel mutants activated by cold (8°C) and menthol (5 mM).

(E) Schematic representation of the chimeras between RbTRPM8 (gray) and XtTRPM8 (red). The responses of wild-type and chimeric TRPM8 channels to cold and menthol are given.

(F) Dose–response curves of TRPM8 orthologs and the chimeric channels in the presence of menthol. Data were fitted to a Hill equation (average ± SEM; n = 3). The chimeric channels are named as X1_X2. X represents the species-specific TRPM8. X1_X2(N) means that the N terminal of X1TRPM8 was substituted by the homologous region of X2TRPM8.
Fig. S3. Site-direct substitutions of XITRPM8.

(A) Representative current traces of XITRPM8 (*Xenopus tropicalis*) and the channel mutants activated by cold (8°C) and menthol (5 mM). (B) The effect of the XITRPM8 mutations for cold or menthol activation. The cold-induced activation of the channel mutants is normalized to that of XITRPM8. The menthol activation of the mutants is normalized to the XITRPM8 activation induced by 5 mM menthol (average ± SEM; n = 3). (C) Summary of the maximum cold response in wild-type XITRPM8 and the point mutations. The currents were normalized to the current amplitude induced by saturating menthol (average ± SEM; n = 3; *P<0.01). (D) Distribution of W177, D377, E438 and L537 on the XITRPM8 structure model.
Fig. S4. Conformational changes of MHR1-3 and pore domain in TRPM8 orthologs.

(A) Representative images of HEK293 cells expressing the MHR1-3 domain of XtTRPM8 incorporating ANAP mutation in the absence or presence of pANAP. Pseudocolors for ANAP are used (Scale bar, 10 μm). (B) Representative ANAP emission spectra measured at 10 and 30°C for each isolated domain. (C) Summary of the shifts in emission peak of ANAP (average ± SEM; n = 3). The species-specific MHR1-3 domains with a large shift in the ANAP emission peak are colored in red. Pa, Protopterus annectens; Rb, Rhinatrema bivittatum; Cm, Chelonia mydas; Pv, Pogona vitticeps; Pb, Python bivittatus; Gg, Gavialis gangeticus; Ge, Gopherus evgoodei. The homologous sites in the TRPM8 orthologs are given as the XtTRPM8 amino acid number. (D) Representative emission peak shifts (top) and currents (middle) of ANAP-incorporated XtTRPM8 upon cooling (bottom).
Fig. S5. Conformational changes at the closed and cold-activated states.

(A) Distribution of pore radii of the XtTRPM8 (Xenopus tropicalis) structural models at closed state and cold-activated state (left). Pore radii were calculated by the HOLE program (right). (B) P166, I360, D413, L453, L537, G959 and T960 sites were mapped onto the XtTRPM8 structure models at the closed (in gray) and cold-activated (in cyan) states, respectively. (C) SASA values of residues at P166, I360, D413, L453, L537, G959, T960 sites at the closed and cold-activated states.
Fig. S6. Cold sensitivity of chimeric TRPM8 channels.

(A) Van’t Hoff plots for the cold-induced currents of TRPM8 mutants shown in Figure 4E. Dotted lines represent the fits of the Van’t Hoff equation. (B) The sidechain hydrophobicity of residues (16) in site 906 positively correlates with the measured ΔH values. Data points were fitted to a linear function (average ± SEM; n = 3).
Table S1. Positive selection sites on *Xenopus tropicalis* TRPM8.

| N°  | lnL₀   | lnL₁   | 2ΔlnL | P value | omega | Positive selection sites              |
|-----|--------|--------|-------|---------|-------|---------------------------------------|
| 5   | -2145.26 | -2139.15 | 12.22 | 4.72E⁻⁴ | 95.82 | 96T*, 106G, 548R, 576Y, 583R*, 585F*, 800P, 954I*, 1109N |

* number of sequences
* present 5% significant level
Abbreviation: lnL, log likelihood.
Table S2. Positive selection sites on *Camelus bactrianus* TRPM8.

| N# | lnL0  | lnL†  | 2ΔlnL | P value | omega | Positive selection sites                  |
|----|-------|-------|-------|---------|-------|------------------------------------------|
| 5  | -4580.24 | -4576.85 | 6.78  | 9.2E-3  | 95.82 | 19G, I252*, 267C*, 765H, 791V, 897R, 915V*, 927S*, 1042A |

* number of sequences
* present 5% significant level
Abbreviation: lnL, log likelihood.
Dataset S1. Primers used in this study to generate TRPM8 chimeras.

Dataset S2. Primers used in this study to generate single-point mutants.

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