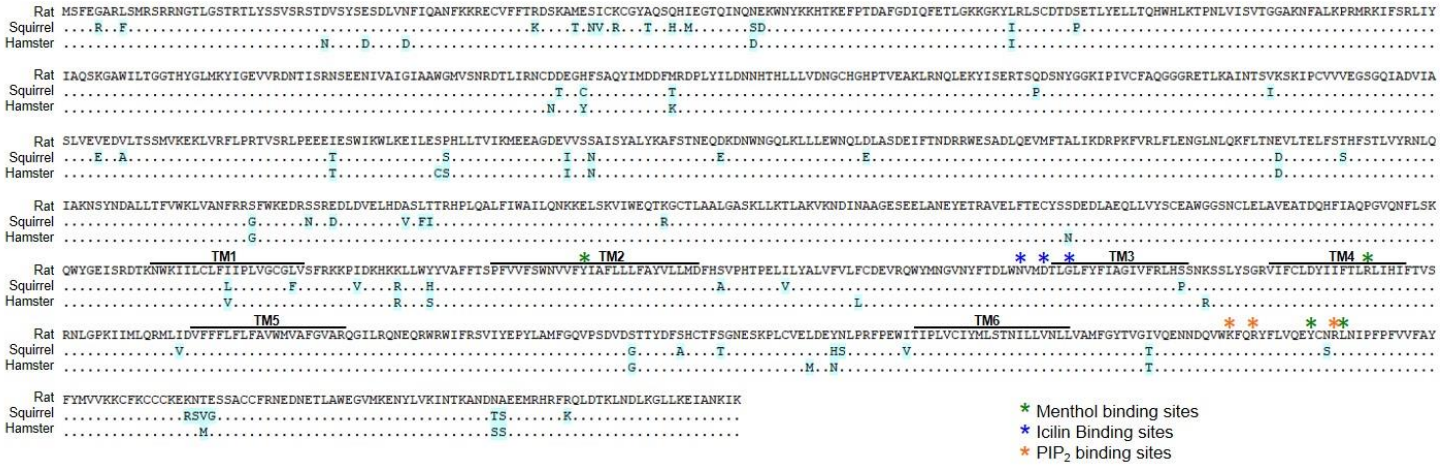
Supplemental data

A



B

Position:	726	762	819	927	946	947
Rat TRPM8	Y	S	S	S	Y	N
Squirrel TRPM8	H	A	P	A	H	S
Hamster TRPM8	S	S	S	S	N	N

Figure S1. (Related to Figure 2) (A) Amino acid alignment of rat TRPM8 (NP_599198), squirrel TRPM8 (MF285605) and hamster TRPM8 (MF285606). sqTRPM8 and hamTRPM8 are 94% identical to each other, and 94% and 97% identical to rTRPM8, respectively. Putative transmembrane domains are denoted by black bars. Asterisks denote amino acids involved in sensitivity to menthol (green asterisks), icilin (blue asterisks) and PIP2 (orange asterisks). (B) Alignment of the six amino acid which modulate cold sensitivity of rat and squirrel TRPM8.

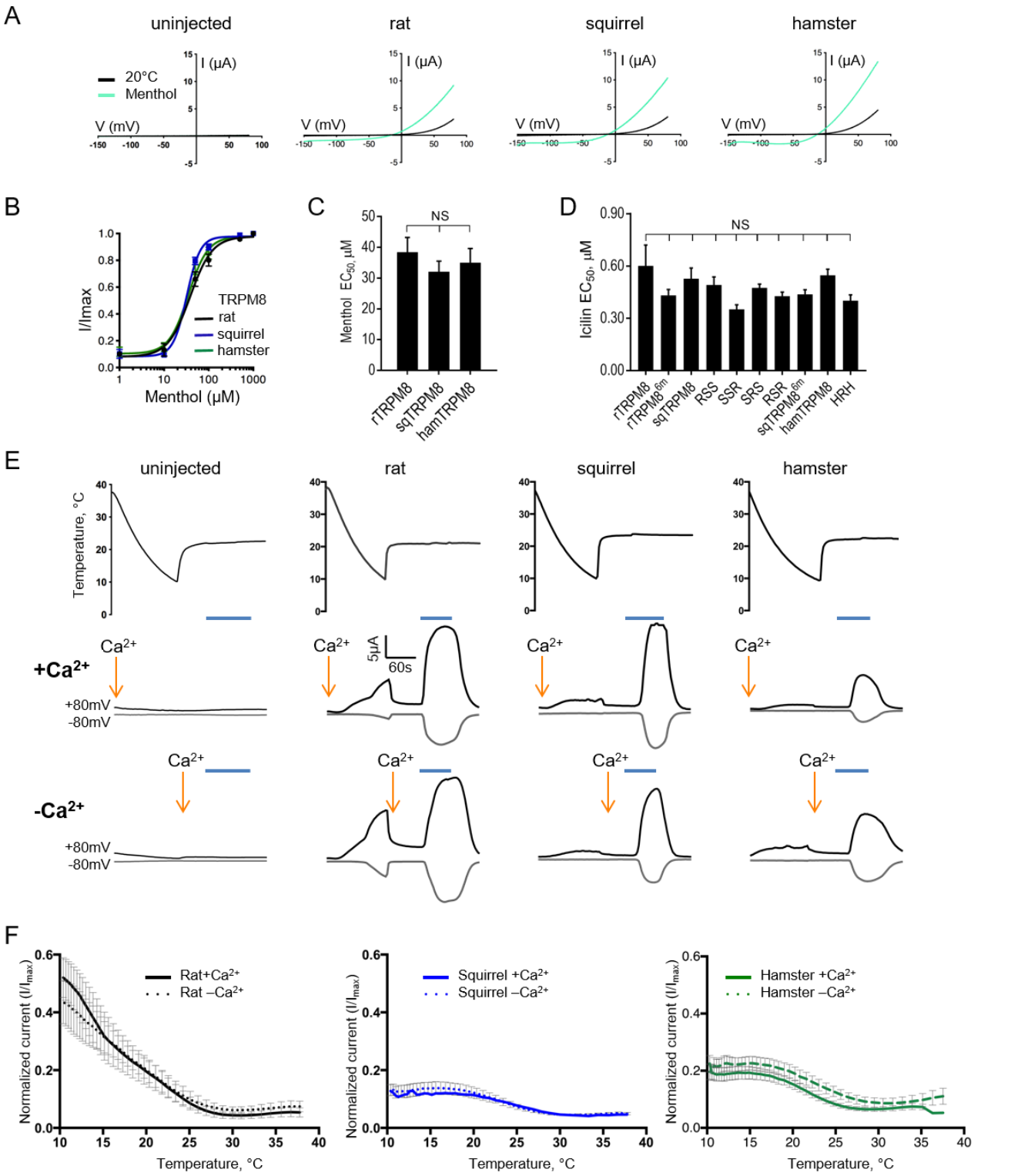


Figure S2. (Related to Figure 3) (A) Exemplar current-voltage plots of rat, squirrel and hamster TRPM8 responses to 500 μM menthol recorded at 20ºC by two-electrode voltage clamp in *Xenopus* oocytes. (B) Menthol dose-response curves for rat, squirrel and hamster TRPM8 orthologues (mean ± SEM, n=6) (C, D) Quantification of EC_{50} for menthol (C) and icilin (D) (mean ± SEM, n=5-8; NS, not significant vs. rat TRPM8, P≥0.05, one-way ANOVA with Dunnett's post-hoc test). (E) Exemplar temperature responses of TRPM8 orthologues recorded by two-electrode voltage clamp with or without extracellular calcium. Note that in the no-calcium condition ($-Ca^{2+}$), 1.8 mM calcium was added back to the solution at the end of the temperature ramp (yellow arrow) to record responses to 1 μM icilin (blue bar). (F) Normalized temperature-response profiles recorded with and without extracellular calcium in oocytes expressing TRPM8 orthologues (mean ± SEM, n≥5).

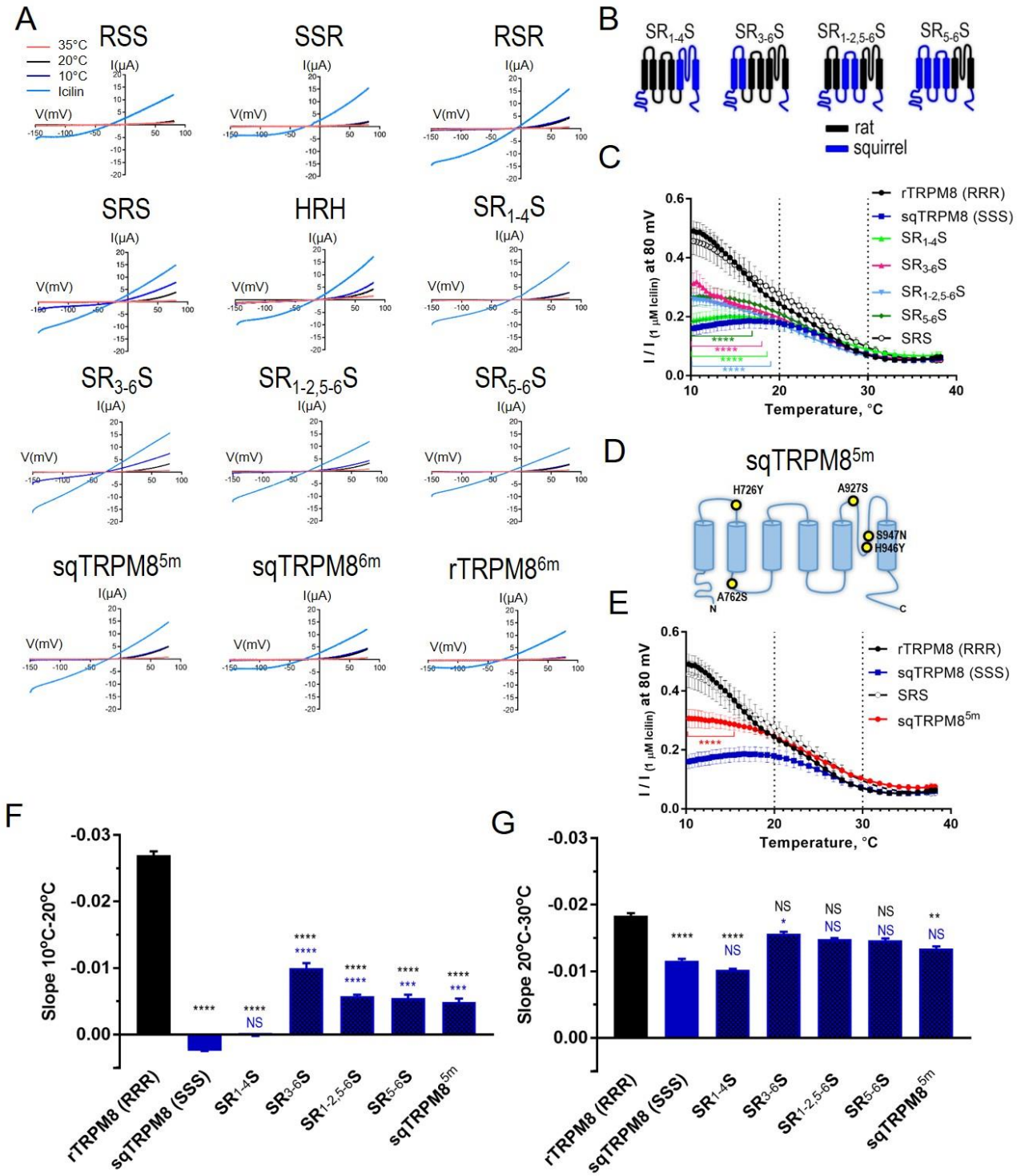


Figure S3. (Related to Figure 4) (A) Exemplar current-voltage plots of the indicated TRPM8 chimeras to cold and 1 μM icilin recorded by two-electrode voltage clamp in *Xenopus* oocytes. (B) Topology diagram of TRPM8 chimeric channels. (C, E) Normalized temperature response profiles for chimeric channels between squirrel, and rat TRPM8 (mean ± SEM, n=9-14; ****0.0001<P<0.05 for data of the same color vs. rat TRPM8, in the range indicated by brackets, not significant (P≥0.05) outside this range, two-way ANOVA with Dunnett's post-hoc test. (D) Topology diagram depicting the locations of the five mutations in sqTRPM8 (yellow circles, sqTRPM8^{5m}). (F, G) Quantification of temperature response steepness (slope) obtained by fitting the data for the 10°C-20°C and 20°C-30°C segments in (C) and (E) to the linear equation (mean ± SEM, n=9-14). NS, not significant, P≥0.05, *P<0.05, **P<0.01, ****P<0.0001 vs. rTRPM8 (denoted by black symbols) or sqTRPM8 (denoted by blue symbols), one-way ANOVA with Dunnett post-hoc test.

Supplemental Experimental Procedures
Resource Table

REAGENT OR RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Xenopus laevis oocytes	Nasco	
Deposited Data		
Thirteen-lined ground squirrel TRPM8 (sqTRPM8)	GeneBank	MF285605
Syrian hamster TRPM8 (hamTRPM8)	GeneBank	MF285606
Thirteen-lined ground squirrel TRPA1 (sqTRPA1)	GeneBank	MG012465
Experimental Models: Organisms		
Thirteen-lined ground squirrel: *Ictidomys tridecemlineatus*	University of Wisconsin at Oshkosh	
Syrian hamster: *Mesocricetus auratus*	Charles River Laboratory	049
Domestic mouse: *Mus musculus* (C57BL/6) wild-type	Charles River Laboratory	027
Domestic mouse: *Mus musculus* (C57BL/6) TRPM8-/-	Dr. Sven-Eric Jordt, Duke University	
Oligonucleotides		
sqTRPM8 cloning FWD CCAGGCAAGATGTCCTTCGAG	This paper	
sqTRPM8 cloning Rev GATGTCCTTCGAGCGTGC	This paper	
hamTRPM8 cloning FWD ATGTCCTTCGAGGGAGCCAG	This paper	
hamTRPM8 cloning Rev TTATTTGATTTTATTAGCAA	This paper	
sqTRPA1 cloning FWD TGTCCTGAGCAGATACAGACAC	This paper	
sqTRPA1 cloning REV ACGGAGTGGCATCAATCAGA	This paper	
RNA *in situ* probe amplification primers:		
TRPM8 FWD CATGAGGAGCAGGAGGAATG	This paper	
TRPM8 Rev GTAGGCAAAGACGACGAAGG	This Paper	
Recombinant DNA		
Rat-TRPM8-pMO	Dr. David Julius's lab (UCSF)	
Squirrel-TRPM8-pMO	This paper	
Hamster-TRPM8-pMO	This paper	
Squirrel-TRPA1-pMO	This paper	
Software and Algorithms		
Prism 7	Graph pad	
ImageJ	NIH	
Pclamp	Molecular Devices	

Contact for Reagent and Resource Sharing
Further information and requests for resources and reagents should be directed to Elena Gracheva (elena.gracheva@yale.edu) and Sviatoslav Bagriantsev (slav.bagriantsev@yale.edu).

Experimental Model and Subject Details

Calcium imaging. Primary neurons were dissected from 3 active squirrels (~1 year old) and 3 mice (8-14 weeks old). Animals were euthanized via CO_2 inhalation followed by decapitation. Dorsal root ganglia were dissected into ice-cold phosphate buffered saline. Tissue was then briefly treated with collagenase P (1mg/mL in HBSS, 15 min, 37°C) followed by 0.25% trypsin (10 min, 37°C). Finally, tissue was suspended in DMEM complete media supplemented with 10% FBS and penicillin/streptomycin and mechanically dissociated using a plastic tipped pipette before being plated on BD Matrigel™ coated coverslips and incubated at 37°C. After neurons were adherent (~2hrs), coverslips were loaded with 10 µM Fura 2-AM (Invitrogen) and 0.02% Pluronic F-127 for 1 hour at 37°C. Images were obtained using Axio-Observer.Z1 inverted microscope (Zeiss) equipped with an Orca-Flash4.0 camera (Hamamatsu) using MetaFluor software (Molecular Devices). For loading and imaging, cells were maintained in physiological Ringer's solution (pH 7.4) containing (in mM): 140 NaCl, 5 KCl, 10 HEPES, 2 $CaCl_2$, 2 $MgCl_2$, and 10 D-glucose. Cold ramps were applied using the SC-20 in-line heater-cooler and CL-200A Bipolar Temperature controller (Warner), with bath temperature monitored via a thermistor situated in the imaging chamber. At the end of imaging, neurons were differentiated from other cell types based on their responses to high K^+ Ringer's solution containing (in mM): 10 NaCl, 135 KCl, 10 HEPES, 2 $CaCl_2$, 2 $MgCl_2$ and 10 D-glucose. Images were sampled at 1 Hz with 25 ms exposure time at 340 nm and 50 ms at 380 nm. The ratioed traces from all icilin-responding neurons were combined by binning per 1 degree ^oC and averaging.

Electrophysiology. Neurons were loaded with Fura-2AM at 37˚C as described above approximately 1 hour before imaging and recording. Neurons with increased intracellular calcium in response to 10 µM icilin were selected for recording. The internal pipette solution consisted of (in mM): 140 CsCl, 2 Mg-ATP, 10 HEPES, 1 EGTA (pH 7.4 with CsOH). External solution contained (in mM): 140 NaCl, 3 KCl, 10 HEPES, 2 CaCl2, 1 MgCl2, 10 glucose (pH 7.4 with NaOH). Neurons were held at -60mV in voltage clamp mode while temperature was decreased from 32.3±0.3˚C (mean±SEM, n=9, range 30-33˚C) to 11.5±0.4˚C, range, 10-14˚C) and back, followed by application of 10 µM icilin. Electrophysiology data were converted from Pclamp format using Taro Tools (Dr. Taro Ishikawa, https://sites.google.com/site/tarotoolsregister/). Neuronal recording and imaging data was analyzed using custom routines written in Igor Pro 6.3 (Wavemetrics, Oswego CA).

Cloning, gene synthesis, mutagenesis, plasmids. Rat *Trpm8* was provided by Dr. David Julius, University of California, San Francisco. The squirrel *Trpm8* orthologue was cloned from cDNA from dorsal root ganglia using the following primers: forward 5'- CCAGGCAAGATGTCCTTCGAG -3'; reverse 5'- GATGTCCTTCGAGCGTGC - 3'. The squirrel *Trpa1* orthologue was cloned from cDNA from dorsal root ganglia using the following primers: forward 5'- TGTCCTGAGCAGATACAGACAC -3'; reverse 5'- ACGGAGTGGCATCAATCAGA - 3'. Hamster *TRPM8* was cloned from cDNA from dorsal root ganglia using following primers: forward 5'- ATGTCCTTCGAGGGAGCCAG-3'; reverse 5'- TTATTTGATTTTATTAGCAA - 3'. The TRPM8 orthologues were cloned into the pMO vector. Point mutations and chimeric channels were generated using the QuickChange Site-Directed mutagenesis kit (Agilent) and overlapping PCR. All constructs were verified by full-length sequencing.

Squirrel TRPM8. The protein sequence of squirrel TRPM8 that we cloned from squirrel dorsal root ganglia is shown below and was deposited to GeneBank under the accession number MF285605.

MSFERARFSMRSRRNGTLGSTRTLYSSVSRSTDVSYSESDLVNFIQANFKKRECVFFTKD
SKATENVCRCGYTQSHHMEGTQINQSEKWNYKKHTKEFPTDAFGDIQFETLGKKGKYIRL
SCDTDPETLYELLTQHWHLKTPNLVISVTGGAKNFALKPRMRKIFSRLIYIAQSKGAWIL
TGGTHYGLMKYIGEVVRDNTISRNSEENIVAIGIAAWGMVSNRDTLIRNCDTEGCFSAQY
IMDDFTRDPLYILDNNHTHLLLVDNGCHGHPTVEAKLRNQLEKYISERTSPDSNYGGKIP
IVCFAQGGGRETLKAINTSIKSKIPCVVVEGSGQIADVIASLVEEEDALTSSMVKEKLVR
FLPRTVSRLPEEETESWIKWLKEILESSHLLTVIKMEEAGDEIVSNAISYALYKAFSTNE
QEKDNWNGQLKLLLEWNQLELASDEIFTNDRRWESADLQEVMFTALIKDRPKFVRLFLEN
GLNLQKFLTNDVLTELFSSHFSTLVYRNLQIAKNSYNDALLTFVWKLVANFRRGFWKEDR
NSRDDLDVELHDVSFITRHPLQALFIWAILQNKKELSKVIWEQTRGCTLAALGASKLLKT
LAKVKNDINAAGESEELANEYETRAVELFTECYSSDEDLAEQLLVYSCEAWGGSNCLELA
VEATDQHFIAQPGVQNFLSKQWYGEISRDTKNWKIILCLFLIPLVGCGFVSFRKKPVDKH
KRLLWHYVAFFTSPFVVFSWNVVFYIAFLLLFAYVLLMDFHAVPHTPELVLYALVFVLFC
DEVRQWYMNGVNYFTDLWNVMDTLGLFYFIAGIVFRLHPSNKSSLYSGRVIFCLDYIIFT
LRLIHIFTVSRNLGPKIIMLQRMLVDVFFFLFLFAVWMVAFGVARQGILRQNEQRWRWIF
RSVIYEPYLAMFGQVPSDVDGTTYDFAHCTFTGNESKPLCVELDEHSLPRFPEWVTIPLV

CIYMLSTNILLVNLLVAMFGYTVGTVQENNDQVWKFQRYFLVQEYCSRLNIPFPFVVFAY
FYMVVKKCFKCCCKERSVGSSACCFRNEDNETLAWEGVMKENYLVKINTKANDTSEEMRH
RFKQLDTKLNDLKGLLKEIANKIK

Hamster TRPM8. The protein sequence of hamster TRPM8 that we cloned from hamster dorsal root ganglia is shown below and was deposited to GeneBank under the accession number MF285606.
MSFEGARLSMRSRRNGTLGSTRTLYSSVSRSTNVSYSDSDLVDFIQANFKKRECVFFTRD
SKAMESICKCGYAQSQHIEGTQINQDEKWNYKKHTKEFPTDAFGDIQFETLGKKGKYIRL
SCDTDSETLYELLTQHWHLKTPNLVISVTGGAKNFALKPRMRKIFSRLIYIAQSKGAWIL
TGGTHYGLMKYIGEVVRDNTISRNSEENIVAIGIAAWGMVSNRDTLIRNCNDEGYFSAQY
IMDDFKRDPLYILDNNHTHLLLVDNGCHGHPTVEAKLRNQLEKYISERTSQDSNYGGKIP
IVCFAQGGGRETLKAINTSVKSKIPCVVVEGSGQIADVIASLVEVEDVLTSSMVKEKLVR
FLPRTVSRLPEEETESWIKWLKEILECSHLLTVIKMEEAGDEIVSNAISYALYKAFSTNE
QDKDNWNGQLKLLLEWNQLDLASDEIFTNDRRWESADLQEVMFTALIKDRPKFVRLFLEN
GLNLQKFLTNDVLTELFSTHFSTLVYRNLQIAKNSYNDALLTFVWKLVANFRRGFWKEDR
SSREDLDVELHDASLTTRHPLQALFIWAILQNKKELSKVIWEQTKGCTLAALGASKLLKT
LAKVKNDINAAGESEELANEYETRAVELFTECYSNDEDLAEQLLVYSCEAWGGSNCLELA
VEATDQHFIAQPGVQNFLSKQWYGEISRDTKNWKIILCLFVIPLVGCGLVSFRKKPIDKH
KRLLWSYVAFFTSPFVVFSWNVVFYIAFLLLFAYVLLMDFHSVPHTPELILYALVFVLLC
DEVRQWYMNGVNYFTDLWNVMDTLGLFYFIAGIVFRLHSSNRSSLYSGRVIFCLDYIIFT
LRLIHIFTVSRNLGPKIIMLQRMLIDVFFFLFLFAVWMVAFGVARQGILRQNEQRWRWIF
RSVIYEPYLAMFGQVPSDVDGTTYDFSHCTFSGNESKPLCVEMDENNLPRFPEWITIPLV
CIYMLSTNILLVNLLVAMFGYTVGTVQENNDQVWKFQRYFLVQEYCNRLNIPFPFVVFAY
FYMVVKKCFKCCCKEKNMESSACCFRNEDNETLAWEGVMKENYLVKINTKANDSSEEMRH
RFRQLDTKLNDLKGLLKEIANKIK

Squirrel TRPA1. The protein sequence of squirrel TRPA1 that were cloned from squirrel dorsal root ganglia is shown below and was deposited to GeneBank under the accession number MG012465.
MKRSLRKMLRPGEKEPQDVVYQGVEDDMDESKDSFKVVFEGNAHRLQNFIKNRRKLSKYDD
TNASPLHHAAAEGQVELMKTIISGSSCAVLNVMDDYGNTPLHWATEKNQVESVKFLLSQGA
NPNLRNGNMMAPLHIAAQGMYNEIIKVLTEHRSTNINLEGENGNTALLTTCAKDNSEALQILL
NKGAKLCKSNKWGDFPVHQAAFSGAKKCMEIILKHGEEHGYSRQSHINFVNNKKVSPLHLAV
QSGDLEMIKMCLDNGAHIELVENGKCMALHFAATQGATEIVKLMISSYSGDSNIVNALDGNC
ETLLHRASLFDHHELAEYLISAGADINSTDSEGRSPLILATASASWNIVNLLLSKGARVDIKDHL
GRNFLHLTVQQPYGLKNLQPEFMQMQHIKELVMDEDNDGCTPLHYACRQGVPVSVNNLLGF
NVSIHSKSKDKKSPLHFAASYGRINTCQRLLQDISDTRLLNEGDLHGMTPLHLAAKNGHDKV
VQLLLKKGALFLSDHNGWTALHHASMGGYTQTMKVILDTNLKCTDRLDEEGNTALHFAARE
GHAKAVALLLSHDAEIILNKQQASFLHIALHNKRKEVVLTTIRSKRWDECLQVFTHHSPSNRC
PTMEMVEYLPECMKVLLDFCMIPSTEDKSCRDYHIEYNFRYLQCPLELTKHTTPIQGVIYEPLS
VLNVMVQHNRIELLNHPVCKEYLLMKWCAYGFRAHMMNLGSYCLGLIPMTLLVVNVKPGM
AFNSTGIINETSDHLEILDSTNSYAIQVCMILVFLSSIFGYCKEVVQIFQQKRNYFLDYNNALEW
LIYTTSIIFVLPLFVNVPANVQWQCGAIAIYFYWMNFLLYLQRFENCGIFIVMLEVILKTLLRST
VVFVFLLLAFGLSFYVLLNIQDAFSSPLLSIIQTFSMMLGDINYRDAFLEPFLRNELAYPFLSFAQ
LIVFTMFVPIVLMNLLIGLAVGDIAEVQKHASLKRIAMQVELHTSLEKKLPLWFLRKVDQKSTI
VYPNRPRGGKILRLFHYLFGSHEIRQEISNTDTCLEIEMLKQKYRLKDLTSLLEKQHELIKLIIQK
MEIISETEDEDNHCSFQDKFKKERLELMSSRWNSVLRAVKTKTQCLEPRP

RNA *in situ* hybridization. Dorsal root ganglia were dissected and fixed overnight in 4% paraformaldehyde in phosphate buffered saline. RNA *in situ* hybridization was performed on cryostat sections (12 μm) using digoxigenin-labelled cRNA probes generated by T7/T3 *in vitro* transcription reaction using *Trpm8* cDNA. Signal was developed with alkaline phosphatase-conjugated anti-digoxigenin Fab fragments according to the manufacturer's instructions (Roche).

Oocyte electrophysiology. Oocytes were purchased from Nasco and maintained at 18°C in ND96 media containing (mM): 2 KCl, 96 NaCl, 20 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES/NaOH pH 7.4 and supplemented with penicillin/streptomycin. cRNA was synthesized by *in vitro* transcription from TRPM8- or TRPA1-containing pMO plasmids linearized with, respectively, *SwaI* or *PmeI* using the mMessage mMachine kit (Ambion), and 2-10 ng RNA was injected per oocyte. Electrophysiological recording were performed by two-electrode voltage clamp (TEVC) 1-4 days post-injection using the OC-725 amplifier. Whole-cell currents were elicited by 3 s voltage ramp from -150 to +90 mV from a holding potential of -80 mV in ND96 pH 7.4 (NaOH), filtered at 1 kHz, sampled at 5 kHz using the Digidata 1440 digitizer and recorded in pCLAMP 10.3 software

(Molecular Devices). Oocytes that displayed currents in excess of 20 μA at 80 mV were discarded from analysis. Cold ramps were applied using the SC-20 in-line heater/cooler and CL-100 Temperature controller (Warner), with bath temperature monitored via a thermistor situated adjacent to the oocyte. Icilin (Sigma-Aldrich) was dissolved in DMSO for a stock concentration of 100 mM, stored at -20°C and diluted to the working concentration prior to experiment. To obtain dose-response curves for icilin, whole-cell currents were measured at 80 mV were fitted to a modified Hill equation: $I = I_{min}+(I_{max}-I_{min})/(1+([C]_{1/2}/[C])^{H})$, where I_{min} and I_{max} are the minimal and maximal current values, respectively, $[C]_{1/2}$ is the half-maximal effective concentration of icilin (EC_{50}), and *H* is the Hill coefficient. Cold responses were recorded from TRPM8-injected oocytes that were exposed to temperature range from 37°C to 10°C in the presence or absence of 1.8mM $CaCl_2$. Recordings at temperatures below 10°C were unstable due to frequent background leak and were not included in the analysis. After cold exposure, the bath was perfused with Ca^{2+}-containing ND96 medium at room temperature, followed by icilin (1μM) in the presence of Ca^{2+}. Cold responses were normalized using maximum icilin value.