Convergent evolution of noxious heat sensing by TRPA5, a novel class of heat sensor in *Rhodnius prolixus*

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ABSTRACT

Insects are ectotherms, and as such, rely on a diverse repertoire of thermoreceptors to monitor environmental temperature and control behavioral thermoregulation. Here, we use structural, phylogenetic, genetic, and functional analyses to show that TRPA5 genes, widespread across numerous insect orders, encode a novel class of noxious heat receptors. We show that in the triatomine bug *Rhodnius prolixus*, the primary vector of Chagas disease, Rp-TRPA5$_2$ differs biophysically and structurally from noxious thermoTRPAs previously described in insects. This includes key changes in the ankyrin repeat domain and the selectivity filter of the channel. *In vitro*, we find evidence that the homo-tetrameric channel is not activated by voltage, but displays high thermosensitivity with an enthalpy change (ΔH) of 72 kcal/mol associated with the channel activation, with a Q_{10} = 25 and $T°_{half}$ = 58.6°C. Structural analyses reveal parallels in the overall ion channel architecture between fruit fly TRPA1 and Rp-TRPA5$_2$; however, functional properties and expression patterns indicate that the role of Rp-TRPA5$_2$ is more similar to that of *Pyrexia* noxious heat receptors found in fruit flies. *Pyrexia* genes have been lost in true bugs, and our findings suggest that the rapidly evolving insect TRPA gene family has given rise to an independent evolutionary origin of a molecular transducer that is responsive to noxious thermal stimuli.

43 **Introduction**

44 Animal thermosensation is critical for performance in fluctuating environments. Changes in
45 environmental temperature are transduced by the sensory system as part of physiological feedback
46 controlling responses such as metabolic homeostasis, feeding, finding suitable habitats, and extreme-
47 heat avoidance [1, 2]. At the molecular level, thermal perception is mediated by the temperature-
48 dependent activation of specific cold- and heat-activated receptors [3, 4]. Although families such as
49 ionotropic receptors (IRs) and gustatory receptors (GRs) have been linked to peripheral innocuous
50 thermosensation in insects [3-6], the transient receptor potential (TRP) receptor family encodes the
51 greatest diversity of thermosensitive channels. TRP receptors are remarkably diverse (TRPA, TRPC,
52 TRPN, TRPM, TRPML and TRPV) and play salient roles as polymodal ion channels responding to
53 chemical, mechanical, and thermal stimuli [7-12].

54 Mammalian TRP channels involved in temperature detection (thermoTRPs) belong to the
55 TRPA, TRPV and TRPM subfamilies (Table 1) and are activated by temperatures from noxious cold
56 to noxious heat [4, 9, 13-16]. In invertebrates, known thermoTRP channels have so far been restricted
57 to the ankyrin TRPA subfamily of genes including *Painless*, *Pyrexia*, *TRPA1*, and *Hs-TRPA* (Fig. 1;
58 Fig. S1) [11, 12]. In *Drosophila melanogaster, Painless*, *Pyrexia* and *Dm-TRPA1* isoforms A, B and D
59 encode receptors that exhibit distinct biophysical properties, cellular expression patterns and
60 temperature activation thresholds ranging from 19°C to 46°C [17-24]. TRPA1 is also a heat–activated
61 TRP sensor in *Anopheles gambiae* (25-37°C), and other mosquitos [25, 26], playing a key role in
62 tuning heat-seeking behavior. Outside the Diptera, TRPA1 has been characterized as a heat-sensitive
63 channel in other insects as it is known to regulate the induction of embryonic diapause in *Bombyx mori*
64 at temperatures above 21°C [27]. The subfamily *Waterwitch* includes receptors responding to stimuli
65 in different modalities from ancestral hygrosensation found in fruit flies [20] to derived heat sensing
66 exhibited by hymenopterans and mediated by the Hs-TRPA subfamily, which diverged following a
67 duplication from *Waterwitch* [12]. Thus, despite the loss of TRPA1 in Hymenoptera, in honeybees,
68 *Apis mellifera* Am-HsTRPA responds to temperatures around 34°C, in parasitoid wasps, *Nasonia*

69 *vitripennis* Nv-HsTRPA activates in response to small temperature differences in the range 8°C to
70 44°C regardless of initial temperatures, and in fire ants, *Solenopsis invicta,* Si-HsTRPA is activated in
71 the range 28-37°C [28, 29]. Notably, the insect TRP ankyrin family has an additional subfamily of
72 unknown function, TRPA5, which is seemingly absent from the fruit fly genome yet found across
73 several other orders of insects [11].

74 Here we deorphanize and characterize an ankyrin TRPA5 ion channel from the "kissing" bug,
75 *Rhodnius prolixus*. Long used as a model organism in studies of insect development and physiology
76 [30], *R. prolixus* (Hemiptera, Reduviidae: Triatominae) has become increasingly relevant for molecular
77 and functional studies. This is primarily explained by its long-term medical and societal impact as a
78 haematophagous vector of *Trypanosoma cruzi*, the causative agent of Chagas' disease [31]. Due to
79 the progressive adaptation of wild triatomine vector species to domestic environments, vector
80 transmission to human populations has increased in recent years [32, 33]. The disease currently
81 affects over 8 million people worldwide, with vector transmission causing around 30,000 new cases
82 yearly [32, 34, 35]. Extensive long-term efforts towards decoding the sensory ecology of triatomines
83 [36-38] have identified olfactory, thermal and environmentally-mediated cues as well as the
84 neuroethology underlying its complex host-seeking behavior [38-45]. Moreover, the annotated *R.*
85 *prolixus* genome [37] and recent transcriptomic studies [44-46] provide detailed expression profiles of
86 candidate sensory receptor genes, including olfactory, ionotropic, pickpocket, and transient receptor
87 potential receptors that can be used to probe the genetic basis of sensory traits [47, 48].

88 In this study, we leverage the rich genomic and transcriptomic resources available for *R.*
89 *prolixus* along with molecular, structural modeling and functional approaches to characterize a TRPA5
90 ion channel enriched in canonical sensory tissues. The biophysical properties of the ion channel
91 demonstrate that *TRPA5* encodes a novel ankyrin type of heat-activated TRP receptor responding to
92 noxious temperatures. Analyses of predicted structures reveal that the channel displays unique
93 features among the ankyrin family, potentially affecting its ion conduction properties despite sharing

94 conserved structural domains with other ankyrin thermoTRPs. These findings may facilitate future
95 studies of agonist responses of TRPA5 to assist in the development of novel genetic tools for vector
96 control efforts.

97 **Table 1**. Vertebrate and invertebrate TRP ion channels involved in thermal transduction.

Nomenclature	Other names	Organism	Expression	TRP family	Thermal treshold*	Activity range	Entropy change (ΔS) cal/mol*K	Activation enthalpy (ΔH) kcal/mol	Q10**	Reference
Vertebrate transient receptor potential (TRP) channels involved in thermosensation										
TRPV1	Vr1	*Rattus norvegicus* (rat), *Homo sapiens* (human)	nociceptor neurons	Ankyrin	≥ 42℃ (40-45)		260 (at 30mV)	90-100	16.8, 20*	Caterina et al 1997, Cheng et al 2012*, Yao et al 2010*
TRPV1(l)	TRPV1- long	*Desmodus rotundus* (vampire bat)	DRG and TG neurons	Ankyrin	40℃				12	Gracheva et al. 2011
TRPV1(s)	TRPV1- short	*Desmodus rotundus* (vampire bat)	TG neurons	Ankyrin	30℃				5	Gracheva et al. 2011
TRPV2	Vrl2	*Rattus norvegicus* (rat), *Homo sapiens* (human)	nociceptor neurons	Ankyrin	≥ 52℃ (50-53)		586	200	20.6	Caterina et al 1999
hTrpv3	Vrl3	*Homo sapiens* (human)	skin keratinocytes	Ankyrin	≥ 33℃ (34-38℃)	33℃~50℃ (Max 41-47℃)	n.d		6.62	Peier et al 2002; Smith et al 2002
hTrpv4	OTRPC4, VR-OAC, Trp12, Vrl2	*Homo sapiens* (human)	skin and dorsal root ganglion neurons	Ankyrin	~ 27-34℃		n.d		9.9+/-3.8	Güler et al 2002; Watanabe et al 2002
TRPM8	CMR1			Melastatin	≤ 23-25℃	0℃-25℃	-384	-112	24	McKemy et al 2002; Brauchi et al 2004;
TRPM3		*Mus musculus* (mouse)	DRG and TG sensory neurons	Melastatin	≥ 43℃				7.2	Vriens et al 2011
TRPC5		*Mus musculus* (mouse), *Homo sapiens* (human)	DRG neurons	Canonical	<37℃->25℃		n.d.	-40		Zimmermann et al 2011
Anktm1	TRPA1	*Mus musculus* (mouse), *Homo sapiens* (human)	DRG neurons	Ankyrin	≤ 17℃	0℃-17℃	-140	-40	6	Story et al 2003
Chicken TRPA1	TRPA1	*Gallus gallus domesticus* (chicken)	DRG neurons	Ankyrin	39.4℃					Saito et al 2014
xtTRPA1	TRPA1	*Xenopus tropicalis* (Western clawed frog)	DRG neurons	Ankyrin	39.7℃				59.24+/-18	Ohkita et al 2012
xlTRPA1	TRPA1	*Xenopus laevis* (African clawed frog)	DRG neurons	Ankyrin	36.2 +/- 0.4℃					Saito et al. 2016
snTRPA1	TRPA1	*Crotalus atrox* (rattlesnake)	TG neurons	Ankyrin	27.6℃				13.7	Gracheva et al. 2010
python TRPA1	TRPA1	*Python regius* (python)	TG neurons	Ankyrin	32.7℃				n.d.	Gracheva et al. 2010
boa TRPA1	TRPA1	*Corallus hortulanus* (boa)	TG neurons	Ankyrin	29.6℃				n.d.	Gracheva et al. 2010
ratsnake TRPA1	TRPA1	*Elaphe obsoleta lindheimeri* (rat snake)	TG neurons	Ankyrin	37.2℃				8.8	Gracheva et al. 2010
Anole TRPA1	TRPA1	*Anolis carolinensis* (green anole)	TG neurons	Ankyrin	33.9℃				45.71+/-6	Saito et al. 2012
A. allogus TRPA1	TRPA1	*Anolis allogus*	Dorsal skin	Ankyrin	33.5 +/- 0.7℃					Akashi et al. 2018
A. homolechis TRPA1	TRPA1	*Anolis homolechis*	Dorsal skin	Ankyrin	36.4 +/- 0.8℃					Akashi et al. 2018
A. sagrei TRPA1	TRPA1	*Anolis sagrei*	Dorsal skin	Ankyrin	33.5 +/- 0.7℃					Akashi et al. 2018
axTRPA1	TRPA1	*Ambystoma mexicanum* (axolotl)	Brain, lung, heart, stomach	Ankyrin	39.7 +/- 1.0℃					Oda et al. 2019
*zTRPA1b****	TRPA1	*Danio rerio* (zebrafish)	Sensory neurons innervating skin and cranial sensory ganglia	Ankyrin	variable < 10℃ (cold); > 25℃ (heat)				8.2+/-0.6	Oda et al. 2016
olTRPA1	TRPA1	*Oryzias latipes* (medaka)		Ankyrin	variable					Oda et al. 2017
trTRPA1	TRPA1	*Takifugu rubripes* (pufferfish)		Ankyrin	variable < 8℃ (cold); > 25℃ (heat)					Oda et al. 2018

Invertebrate transient receptor potential (TRP) channels involved in thermosensation										
*ceTRPA1*****		*Caenorhabditis elegans* (nematode)	neurons, muscle, intestine, and epithelial cells		≤ 17℃					Reviewed in Laursen et al. 2015
Painless	dAnktm1	*Drosophila melanogaster* (fruit fly)		Ankyrin	~39-42℃					Tracey et al 2003; Sokabe et al. 2008
Pyrexia	Pyx-PA and Pyx-PB	*Drosophila melanogaster* (fruit fly)	embryos: peripheral nerves and central nerves, multidendritic neurons in larval epidermis;adults: sensory neurons around bristle eyes, britsle neurons along thorax, neurons in maxillary palps, proboscis, antennae	Ankyrin	≥ 40 ℃				Pyx-PA 18.145; Pyx-PB 15.329	Lee et al. 2005; Neely et al. 2011
dTRPA1(A)	TrpA1-RI (Prom B, ex10a), dTrpA1, dANKTM1, DmTRPA1	*Drosophila melanogaster* (fruit fly)	larval sensory neurons, adult proboscis	Ankyrin	27-29℃				9	Viswanath et al. 2003; Rosenzweig et al. 2005; Hamada et al 2008; Kang et al. 2012
dTRPA1(D)	TrpA1-RG (Prom A, ex10a), dTrpA1, dANKTM1, DmTRPA1	*Drosophila melanogaster* (fruit fly)	larval nociceptors	Ankyrin	≥ 46 ℃				116	Zhong et al. 2012
AgTRPA1		*Anopheles gambiae* (African malaria mosquito)	antennae, head	Ankyrin	25.2℃					Wang et al. 2009
AsTRPA1(A)		*Anopheles stephensi* (Asian malaria mosquito)	antennae, head	Ankyrin	30.3 +/- 0.9℃				14,5	Li et al. 2019
AaTRPA1(B)		*Aedes aegypti* (yellow fever mosquito)	antennae, head	Ankyrin	32 +/- 0.8℃				20,7	Li et al. 2019
CpTRPA1(A)		*Culex pipiens pallens* (northern house mosquito)	antennae, head	Ankyrin	21.8 +/- 0.7℃				61,2	Li et al. 2019
TRPA1		*Bombyx mori* (silk moth)		Ankyrin	21.6℃				20,5	Sato et al. 2014
hsTRPA	AmhsTRPA	*Apis mellifera* (honeybee)	antennae	Ankyrin	33.9 +/-0.6℃				17.2+/-4.0	Kohno et al. 2010
Rp-TRPA5$_2$	Rp-TRPA5B	*Rhodnius prolixus* (kissing bug)	head, rostrum, legs, antennae	Ankyrin	53℃; Thalf 58.6℃	53-68℃	274	72	25	*this study*

* Activation threshold temperature as determined in heterologous expression systems
** Q10 is the fold current increase over 10 ℃ increase as a measure of sensitivity, the higher Q10 value, the more sensitivity to heat the channel

Results and discussion

TRPA5 genes are ancient ankyrin receptors found across many insect orders but lost in Diptera

To start investigating the molecular basis of thermosensation in Rhodnius, we first reanalyzed the genome annotations (Version RproC3.3) complemented with available transcriptomic resources (*see Methods*) to gain insights into gene variation and genomic architecture within the TRP ankyrin family. Genomes of triatomines [37] and additional surveyed hemipteran species (Table S1) all lack an ortholog to *Pyrexia* (*Pyx*) TRP but possess one gene copy of the three canonical ankyrin TRP genes: *Waterwitch* (*Wtrw*), *TRPA1* and *Painless* (*Pain*) (Fig. 1A, Fig. S1). Rhodnius *TRPA1* and *Painless* exhibit a wide mRNA tissue distribution (Fig. 1B) that potentially indicates a canonical role in thermosensation, similarly to TRPAs expression patterns in other insect species [27-29, 49].

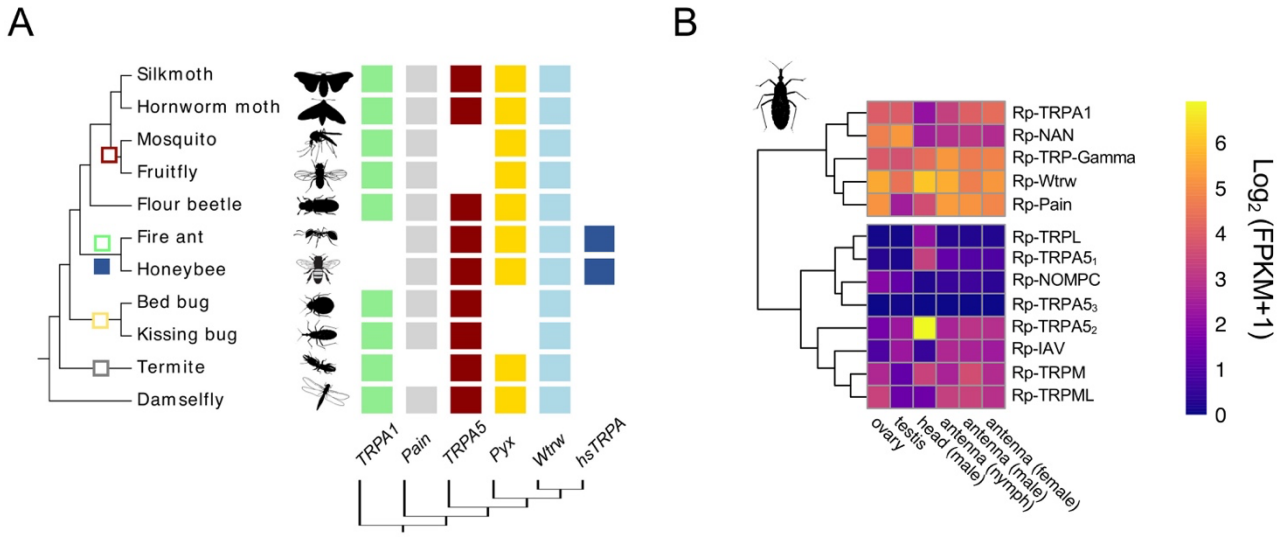

Figure 1. A. Phylogenetic reconstruction of the ankyrin TRP (TRPA) channel family in representative insect species. TRPA5 channels are present across insect Orders but absent from dipteran genomes (see also Fig. S1 and S2). Gene abbreviations: *Painless (Pain), Pyrexia (Pyx), Waterwitch (Wtrw*), hymenopteran-specific TRPA (*hsTRPA*). Silkmoth, *Bombyx mori*; Hornworm moth, *Manduca sexta*; Mosquito, *Anopheles gambiae*; Fruit fly, *Drosophila melanogaster*; Flour beetle, *Tribolium castaneum*; Fire ant, *Solenopsis invicta*; Honeybee, *Apis mellifera*; Bed bug, *Cimex lectularis;* Kissing bug*, Rhodnius prolixus*; Termite, *Zootermopsis nevadensis*; Bluetail Damselfly, *Ischnura elegans.* **B.** Phylogenetic relationships of TRP genes in *R. prolixus* and their corresponding

116 expression levels across tissues in published transcriptomic data. Heat maps compare the expression levels
117 across tissues and developmental stages. Expression levels are represented as Log_2 FPKM +1 and depicted
118 with a gradient color scale. Gene models are based on genomic annotations [37], *de novo* transcriptome
119 assembly [44] and manual annotation of gene models not annotated in the two aforementioned studies (see
120 *Methods*).

121 Our scaffold-mapping analysis of three *TRPA5* isoforms previously annotated in *R. prolixus* [37] shows
122 that the predicted-isoforms A and B map to different genomic locations, and consist of two physically
123 close tandem-duplicate loci, whereas predicted-isoform C maps to a distinct scaffold. Therefore,
124 TRPA5A, TRPA5B and TRPA5C are hereafter referred to as three distinct loci, $TRPA5_1$, $TRPA5_2$, and
125 $TRPA5_3$. Intrigued by the finding of multiple *TRPA5* gene copies, we performed an extensive *TRPA5*
126 gene search across annotated genomic and transcriptomic datasets available for the insect Orders
127 Anoplura, Diptera, Coleoptera, Hemiptera, Hymenoptera, Isoptera, Lepidoptera, Odonata and
128 Thysanoptera. Our phylogenetic reconstruction shows that the TRPA5 ankyrin subfamily is completely
129 absent in all surveyed dipteran genomes (Fig. S2). TRPA5 orthologs were nonetheless found
130 spanning the orders Lepidoptera, Coleoptera, Hymenoptera, Hemiptera, Isoptera and Odonata (Fig.
131 S2), including remarkable group-specific expansions such as those in the fire ant, *Solenopsis invicta*
132 [29], the damselfly *Ischnura elegans*, the tobacco hornworm moth *Manduca sexta*, and several
133 hemipterans (Fig. S2). In addition to lineage-specific expansions through duplications, complex
134 alternative splicing also seems to play a role in TRPA5 functional diversification [50]. Altogether, our
135 large-scale phylogenetic analyses recapitulate that the insect TRPA ankyrin family comprises five
136 rapidly evolving clades consisting of *Waterwitch* (and HsTRPA1), *Pyrexia* (presumably lost in
137 Hemiptera), *TRPA5* (presumably lost in Diptera), *TRPA1* and *Painless* (Fig. 1A, SI File 1, Table S1).

138 Prior to interrogating a possible role for Rhodnius TRPA5 in thermosensation, we asked whether these
139 TRPs are expressed in sensory tissues. Whereas Rp-$TRPA5_1$ and Rp-$TRPA5_3$ mRNAs are expressed
140 at low or below detection thresholds across tissues (Fig. 1B), Rp-$TRPA5_2$ mRNA appears to be the
141 most abundant TRPA5 transcript in adult Rhodnius. Rp-$TRPA5_2$ is significantly enriched in adult male

142 and female heads (Fig. 1B, Fig. S3, Table S5). We further examined expression profiles of Rp-TRPA5$_2$
143 via quantitative PCR of additional canonical sensory tissues. Rp-TRPA5$_2$ is abundant in the rostrum
144 and legs and expressed at lower levels in antennae (Fig. 1B, Fig. S3), a first indication in line with a
145 possible role in thermosensation.

146 ***Rhodnius TRPA5$_2$ exhibits unique structural features in the Ankyrin Repeat Domain and the***
147 ***selectivity filter***

148 To visualize structural features of Rhodnius ankyrin TRP homologs, we used DeepMind's protein
149 structure prediction software AlphaFold 2.0 [51-53]. Monomeric structures of Rhodnius TRPA1,
150 Painless, Waterwitch, and TRPA5$_2$ were modeled, as well as a tetrameric model of the Rhodnius
151 TRPA5$_2$. We then performed pairwise comparisons of the Rhodnius orthologs to their Drosophila
152 correspondent monomeric structures for TRPA1, Painless and Waterwitch, and compared Rp-TRPA5$_2$
153 to Dm-Pyx separately (Fig. 2). Each monomeric prediction is presented using a coloring scheme
154 reflecting a structural reliability measure (Fig. 2A) next to a rainbow representation running from the
155 N- to the C-terminus (Fig. 2B).

156 All Rhodnius and Drosophila ankyrin TRP monomeric structures shared several expected features
157 with the cryo-EM structure of HsTRPA1 [54], including the N-terminal ankyrin repeat domain (ARD),
158 six transmembrane α-helices (S1-S6), and a region corresponding to the allosteric nexus of Hs-TRPA1
159 connecting the ARD and the transmembrane region [54]. The C-terminal region features at least one
160 α-helix, which together with the corresponding helices from the other subunits, most likely form a
161 coiled-coil in the tetramer as seen in the solved TRPA1 structures. Furthermore, the recently published
162 structure of Dm-TRPA1-A in state 1 confirms distinct predicted features in our model, including the
163 interfacial helix and the interaction between AR12 and the region C-terminal of the coiled-coil helix
164 [55]. However, without the constraints of the other monomers and the interactions that would force the
165 C-terminus into the coiled-coil, some of the helices are oriented in unrealistic directions while the
166 secondary structure remains plausible.

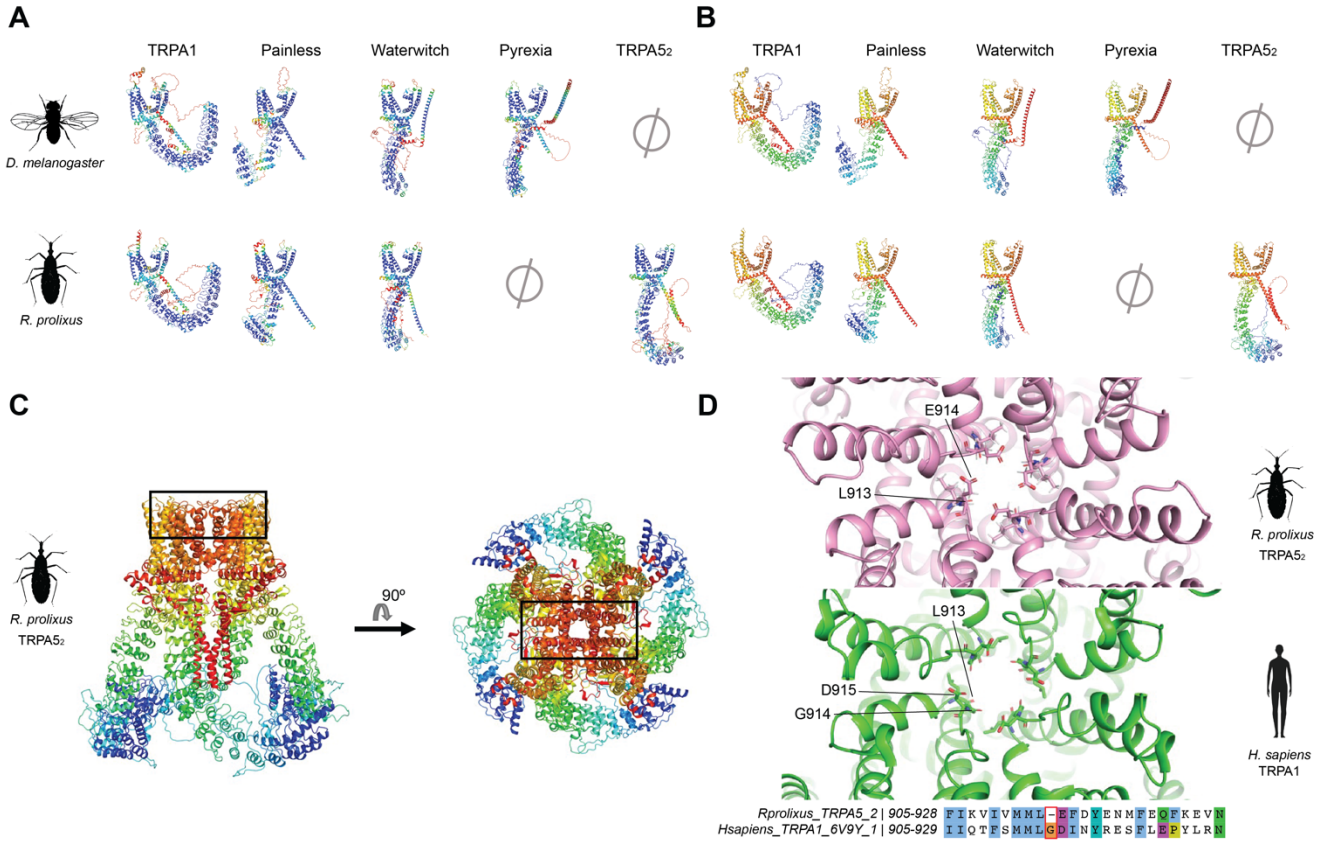

Figure 2. Predicted tetrameric structure of Rp-TRPA5$_2$ alongside monomers of all TRPA homologs in *Drosophila melanogaster* and *Rhodnius prolixus*. **A.** TRPA monomers colored by pLDDT score from the AlphaFold modeling. Blue represents a plDDT score of more than 90, reflecting a high confidence. **B.** TRPA monomers colored by chain bows, with the N-terminus in blue and the C-terminus in red. **C.** Tetrameric model of *R. prolixus* TRPA5$_2$, colored as chain bows as in B. The black box indicates the location of the selectivity filter shown in D. **D.** Top view of the selectivity filter of the pore of *R. prolixus* TRPA5$_2$ (top) and human TRPA1 (*H. sapiens*, pdb:6V9Y) (bottom). Three important residues identified by Zhao et al [56]– L913, G914 and D915 – are marked in TRPA1. The equivalent residues L913 and E914 are marked in TRPA5$_2$. The sequence alignment shows the selectivity filter. Note the glycine is absent in TRPA5$_2$ (see also Fig. S2).

Since dipteran and hemipteran insects lack *TRPA5* and *Pyrexia*, respectively, we asked if the two channels may occupy homologous structural and functional niches. From a pairwise overall comparison of the reliable monomeric models, despite the conserved regions mentioned above, Rp-TRPA5$_2$ appears to deviate from Dm-Pyrexia in three regions: the ARD, the transmembrane domain, and the pore helices that flank the selectivity filter (Fig. S4). First, Rp-TRPA5$_2$ possesses 14 AR

181 compared to 9 in Dm-Pyrexia, and displays longer loops, compared to Dm-Pyrexia and all other
182 channels, including between the third and the fourth ankyrin repeats, within the fifth ankyrin repeat,
183 and between the fifth and the sixth ankyrin repeats, counting from the N-terminus. This observation is
184 interesting as ankyrin repeats are 31-33 residue protein motifs consisting of two α-helices connected
185 by a ß-turn that occur in tandem arrangement to form ARDs critical for physiological processes [57],
186 including a previously suggested role in thermal activation sensitivity [58, 59].

187 Hence, in vertebrates, two regions of 6 ARs each in the snake TRPA1 (AR3-8; AR10-15) have been
188 shown to revert the channel thermal sensitivity by conferring heat-sensitivity to a chimeric AR human
189 TRPA1 (Hs-TRPA1) [60]. Furthermore, transfer of a part of the ARD from Dm-TRPA1 (AR10-15), a
190 region shown to control thermosensitivity in the fruitfly TRPA1, to Hs-TRPA1 also produced a heat-
191 sensitive Hs-TRPA1 [60]. The temperature-dependent dynamics of the ARD has also recently been
192 investigated in the TRPV1 channel, demonstrating that the ARD undergoes structural changes at
193 similar temperatures that lead to TRPV1 activation, which suggested a potential role in the
194 temperature-dependent structural changes leading to the channel opening [58]. The N-terminus region
195 of mosquito TRPA1 also seems to be quite critical for heat-sensitivity [19]; however, there have been
196 contradicting data for TRPA1, both from human and mosquito, arguing that additional regions
197 controlling thermosensitivity are located outside the ARD [26, 61]. Another interesting feature is the
198 disruption in the ankyrin repeat stacking between the fifth and the sixth ankyrin repeat in both Rhodnius
199 and Drosophila Painless, which is not seen in Dm-Pyrexia and Rp-TRPA5$_2$. This breaking point
200 coincides with the resolved N-terminal end of the recently reported structure of Dm-TRPA1-A in state
201 2, which is suggested to represent a temperature sensitized, pre-opened conformation of the channel
202 [55]. Although additional functional evidence that ARDs may play a general role in insect TRP
203 thermosensitivity is needed, these differences between Painless, Pyrexia and TRPA5 hint at a similar
204 activation mechanism in Painless as proposed for TRPA1, yet potentially distinct from the activation
205 mechanism of Dm-Pyrexia and Rp-TRPA5$_2$. In contrast, the shape of the ARD in Dm-Pyx, Dm-Wtrw

206 and Rp-Wtrw all exhibit remarkable similarities compared to that of Rp-TRPA5$_2$ (Fig. 2A) and the
207 predicted number and position of ankyrin repeats also appears remarkably conserved between *R.*
208 *prolixus* and *D. melanogaster* for TRPA1, Painless, and Wtrw (Table 2) suggestive of potentially
209 conserved interspecific functions for each Rhodnius ortholog of the latter three channels.

210 Second, looking at the selectivity filter and the upper gate of the Rp-TRPA5$_2$ tetramer model (Fig. 2C
211 and D), one conserved glycine (Gly914) is notoriously absent in Rp-TRPA5$_2$ (Fig 2D). This is striking
212 as it is conserved in most other TRPAs, and either conserved or substituted for Serine or Threonine
213 in most other non-hemipteran TRPA5 proteins (Fig. S2). Comparing it to a structure of Hs-TRPA1
214 published by Zhao et al. (PDB:6V9Y) [56], the Leu913 and Glu914 in Rp-TRPA5$_2$ seem to remain
215 largely in the same locations as Leu913 and Asp915 in Hs-TRPA1, despite the shorter pore loop. The
216 main difference therefore is that there are only two carbonyls in the pore loop of Rp-TRPA5$_2$, as
217 opposed to three carbonyls in Hs-TRPA1. Although the difference is smaller than anticipated, since
218 Gly914 is suggested to be important in gating, and lies in the location of the selectivity filter, this may
219 affect permeation properties of Rp-TRPA5$_2$.

220 Finally, a difference in the lower gate can be seen where the Hs-TRPA1 structure is much narrower
221 than that of Rp-TRPA5$_2$, and where the pore of the Rp-TRPA5$_2$ model is blocked by the position of the
222 sidechain of Glu914. Since AlphaFold is better at modeling backbone folding than individual
223 sidechains, the significance of this finding may be limited, but the Rp-TRPA5$_2$ model features a
224 narrower pore than Hs-TRPA1 resolved in a closed conformation in complex with an antagonist [56].

225 Our findings that Rp-TRPA5$_2$ shares several conserved features of ankyrin TRPs, as well as unique
226 structural novelties, raises the question as to whether Rp-TRPA5$_2$ may have undergone selection to
227 fill the same function as *Pyrexia*, or whether its distinctive structural features may underlie a new type
228 of thermosensitivity phenotype. If so, it would be interesting to determine whether the heat activation
229 of the channel may be affected.

230 **Table 2**. Number of Ankyrin repeats observed in each monomer structure of thermos TRPA of *Rhodnius prolixus*
231 and *Drosophila melanogaster.*

	Number of Ankyrin repeats	
Name	*Rhodnius prolixus*	*Drosophila melanogaster*
TRPA1	17	17
Painless	10	10
Waterwitch	9	10
Pyrexia		9
$TRPA5_2$	14	

232

233 ***$TRPA5_2$* encodes a novel class of thermosensitive insect receptor**

234 In order to demonstrate a potential role of candidate $TRPA5_2$ as a thermosensitive ion channel, we
235 optimized an *in vitro* cell-based platform to record temperature-elicited currents from HEK293T cells
236 under whole-cell patch-clamp configuration. We transiently expressed a bicistronic T2A-fluorescent
237 marker cassette [62] together with the candidate TRP channel, which localized well to the plasma
238 membrane (Fig. S5). We delivered a fast heat stimulus by coupling an infrared laser diode fiber optic
239 [63] to a PID controller that conveyed millisecond current pulses (Fig. S6).

240 At the molecular level, non-denaturing SDS-Page analysis showed that Rp-$TRPA5_2$ assembles
241 similarly to other TRPs as a membrane-bound tetramer when expressed in HEK293T cells (Fig. S5).
242 To validate the infrared (IR) patch clamp system, we first transiently expressed two known
243 thermoTRPs, the rat TRPV1 (rTRPV1) and fruit fly TRPA1 isoform D (Dm-TRPA1-D) (Figs. S6, S7),
244 both of which formed expected homotetrameric structures (Fig. S5). First, we used the ionic current
245 increments through the open patch pipette (holding potential -2 mV), to calculate the temperature

246 changes associated with the different laser intensities. Typically, the current pulses were set to result
247 in temperature increments at the cell membrane in the range of 23.5-71.7°C. A similar laser stimulation
248 protocol led to marginal whole-cell current changes in non-transfected cells (Fig. 3A, Fig. S7A-B).
249 Compared to non-transfected cells, we then observed a strong increase in the current amplitude of
250 cells expressing rTRPV1 (Fig. 3B, Fig. S7C-D) with an enthalpy change associated with the activation
251 of 88.3±9.4 kcal/mol, which is comparable to the published enthalpy values obtained using millisecond
252 temperature jumps of ΔH = 85 kcal/mol, $T_{1/2}$ = 47.5°C for rTPRV1 (calculated from enthalpy and
253 entropy values for steady-state activation from Yao et al. 2010, see Table 1) [63-65]. A temperature-
254 induced activation response was also observed for the heat activated fruit fly channel, Dm-TRPA1-D,
255 for which in our more precise setup, at 46.3°C [22], the open probability (Po) of the channel is about
256 10% (Po=0.1), corroborating a noxious activation temperature > 42°C [22]. Assuming complete
257 activation by temperature of this channel (Po=1), which was not measured in previous studies due to
258 limitations in the maximum temperature to which the Dm-TRPA1-D channel could be subjected, the
259 activation process is characterized by an enthalpy change ΔH= 68.7±13.1 kcal/mol and $T_{1/2}$ = 53.5°C
260 (Fig. 3C, Fig. S7E-F). These results demonstrate highly consistent biophysical properties of known
261 thermoTRP channels evaluated with our laser-based delivery method, using the modified C-terminus
262 in our expression cassette.

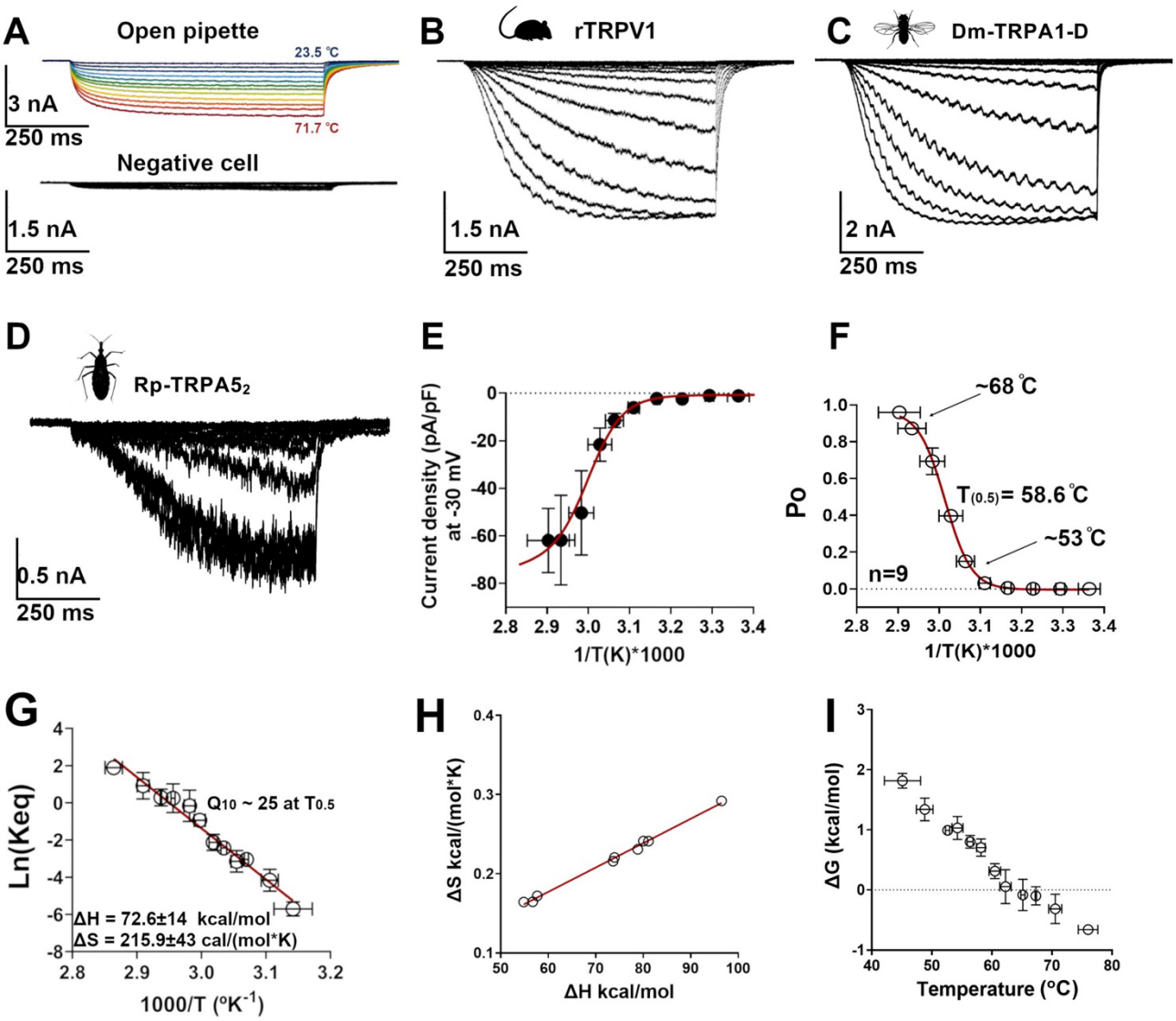

Figure 3. Thermodynamics of Rp-TRPA5$_2$ temperature-activated currents. A. Activation time course of temperature for the open pipette calibration (upper panel), and baseline current traces for a control cell (non-transfected). During each voltage pulse, a temperature step of 700ms was presented from room temperature to 71°C. **A.** Open patch-clamp pipette current traces in response to increasing voltage pulses (10 - 23 mV at 1 mV voltage inputs). **B.** Whole-cell currents evoked by temperature at -30mV of HEK293T cells expressing rat TRPV1, the heat-activated mammalian vanilloid thermo-TRP channel. **C.** Whole-cell currents evoked by HEK293T cells expressing Dm-TRPA1-D. **D**. Whole-cell currents evoked by HEK293T cells expressing Rp-TRPA5$_2$ under whole-cell configuration patch-clamp, held at -30 mV (n=9). Negative currents indicate that Rp-TRPA5$_2$ is a cationic channel. Data are presented as means ± standard errors. **E**. Current-Temperature relationship for Rp-TRPA5$_2$ where current density is plotted after normalization by cell membrane capacitance

273 and scaling to the mean maximum. **F.** Fraction of Rp-TRPA5$_2$ channels in the open state (Open probability, P_o)
274 as a function of the temperature. The Po vs 1/T was fitted to a Boltzman function with the midpoint of activation
275 ($T_{1/2}$) reached at 58.6 °C. **G** Van't Hoff plot estimates of Rp-TRPA5$_2$ with an activation enthalpy of the
276 endothermic transition at 92 kcal/mol and an entropic change associated to the temperature activation process
277 at 274 cal/mol*K. at -30 mV as a function of the temperature [63]. **H**. Coupling between enthalpic (ΔH) and
278 entropic (ΔS) changes for each one of the experiments recorded **I**. ΔG vs. temperature plot for Rp-TRP5A$_2$
279 channels, temperature activation is associated with small ΔG (free energy) changes, as reported for other
280 families of mammalian thermo receptors. ΔG was calculated as -RT*ln(Keq) [66].

281 Finally, when holding the membrane potential at -30mV in patched mRuby2-expressing cells
282 transfected with Rp-TRPA5$_2$, whole cell currents were evoked by temperature steps from 53°C to 68°C
283 (Fig. 3D, 3E). The average temperature for the activation "threshold" was 53°C, defined as Po = 0.1
284 calculated from the Van't Hoff plots. The channel opening appeared to saturate at 68°C (Po=0.9) (Fig.
285 3F), with a $T_{1/2}$ = 58.6°C. The steady-state parameters of activation were calculated from the current
286 at the end of the 700 ms temperature pulse. The current density versus temperature relationship (Fig.
287 3E) indicates that the opening of Rp-TRPA5$_2$ involves an activation enthalpy of approximately 72.6±14
288 kcal/mol (Fig. 3G). Similar large enthalpy changes ranging from 60 to 200 kcal/mol are involved in the
289 opening of TRPM8, TRPV1, and TRPV2 [9], supporting that the TRPA5 ion channel activity is
290 extremely temperature-dependent with high enthalpy change associated with the channel opening.
291 The large entropy value further indicates that the channel transits between a highly ordered closed
292 state and a strongly disordered open configuration, similarly to TRPV1 (316 cal/mol·K at -60mV).
293 Altogether, these results demonstrate that Rp-TRPA5$_2$ acts as an insect thermoTRP receptor of
294 noxious heat *in vitro*.

295 Based on the open probability (Po), we calculated a Q_{10} value of ~ 25, which is in the range of
296 characterized noxious vertebrate (rTRPV1 Q_{10} = 16.8; rTRPV2 Q_{10} = 20.6) and invertebrate
297 thermoTRPs (fruit fly Pyrexia Q_{10} = 18.2) (Table 1). From a thermodynamic point of view, many TRP
298 ion channels are modulated by temperature [67], but our results clearly support that TRPA5$_2$ belongs
299 to a restricted category of thermoTRPs as it is directly activated by temperature as the sole stimulus

300 [8, 66]. Rp-TRPA5$_2$ appears to be activated in a higher noxious range compared to all known
301 invertebrate thermoTRPs thus far (Fig. 1A, Table 1, Fig. S2) including the fruit fly Painless and TRPA1
302 channels that mediate thermal nociceptive escape through larval mdIV neurons at temperatures above
303 40°C and 46°C, respectively [17], or Pyrexia channels that induce paralysis in adult flies upon
304 exposure to 40°C [18]. In mammals, only TRPV2 contributes to highly noxious (>52 °C) heat sensing
305 [64, 68, 69].

306 ThermoTRP channels can integrate voltage and temperature allosterically [67]. We aimed to establish
307 the temperature sensitivity of the channel directly from the Van't Hoff plot, and not from the influence
308 of temperature on the voltage activation process, allowing us to establish the thermodynamics of
309 temperature activation process independently from other stimuli. Using fast temperature jumps, we
310 could activate Rp-TRPA52 channels directly, validating that the channel is a thermoTRP in the noxious
311 temperature range. In addition, similarly to rTRPV1 and Dm-TRPA1-D, Rp-TRPA5$_2$ has large enthalpy
312 changes related to the channel activation, which is related to the high sensitivity of these channels to
313 temperature changes. In rTRPV1, thermal activation at +60 mV causes only a relatively small enthalpy
314 change (30 kcal/mol) compared to 100 kcal/mol when rTRPV1 is activated at -60 mV, representing a
315 three-time increase in temperature sensitivity at negative voltages [63]. Similarly to rTRPV1, Rp-
316 TRPA5$_2$ shows almost no heat-elicited activity at depolarized potentials (> 0mV), but a robust response
317 at negative voltages (-30mV). Although the temperature activation for Rp-TRPA5$_2$ appears very similar
318 to Dm-TRPA1-D and rTRPV1, the activation kinetics of Rp-TRPA5$_2$ is slower compared with these
319 channels, which could be due to intrinsic molecular interactions influencing the transition between
320 closed and open states [67], in light of our observations of several key defining structural differences.
321 This difference in the opening kinetics has been observed before for other thermoTRPs, like the
322 temperature-dependent HsTRPV3, which exhibits a high temperature sensitivity, comparable to
323 hTRPV1, but presents slower kinetics [65, 70].

324 **TRPA5's physiological role as a noxious heat sensor**

325 Sensory receptors in the same clades are often tuned to detect a stimulus over a discrete
326 window of intensities, enabling the recognition of physiologically relevant cues over a wide dynamic
327 range [1, 3, 71]. The TRP Ankyrin family is an excellent example of this pattern, as distinct, yet closely
328 related channels account for thermal responsiveness over a range from innocuous to noxious heat [4,
329 12]. In addition, orthologous thermoTRPs often have different activation temperatures, and this has
330 been postulated to reflect functional adaptive evolution to different optimal temperatures, coordinating
331 thermoregulatory behaviors such as host seeking, thermal avoidance, and tracking of optimal
332 temperatures [72]. Our findings on the temperature activation of Rp-TRPA5$_2$, a TRPA clade not
333 functionally characterized, clearly support a role for this previously orphan gene as a molecular sensor
334 in the noxious heat spectrum. Notably, a known noxious heat receptor in Diptera, *Pyrexia,* is missing
335 from *Rhodnius*, while a TRPA5 ortholog has not been found in flies and mosquitoes*,* raising the
336 interesting possibility that convergence and functional redundancy might account for the evolutionary
337 patterns of differential gain and retention of thermoTRPs in insects.

338 Consistent with a role as a noxious thermosensor, quantitative analyses show that Rp-TRPA5$_2$
339 is expressed broadly across tissues, with high levels detected in the head, rostrum, legs, thorax,
340 abdomen and to a lesser extent in the antennae (Fig. 1, Fig. S3). By qPCR, we detected the highest
341 expression in the head (Fig. S3), in agreement with previous transcriptomic analyses [37] and
342 remarkably similar to the expression pattern of *Pyrexia* in the fruit fly [18]. Noxious environmental
343 temperatures are extremely common in natural environments of small insects with low thermal
344 capacity, thus, detecting and avoiding heat is critical to prevent injury. Temperature distributions vary
345 widely for natural objects. For instance, dry and moderately gray-colored or dark objects such as tree
346 bark or rocks easily reach temperatures above 50°C [73]. If the humidity level is high, and radiative
347 cooling of the sky is not effective, the same objects can reach temperatures above 60°C in the full
348 sun. For example, temperatures of dry leaf substrates on the ground can exceed 50°C in full sun since
349 they do not undergo evaporative cooling, which would typically prevent a leaf's surface temperature
350 from going above 40°C. In lab simulated natural environments and in field thermal imaging studies,

351 insects can reach 60°C under full sun with high humidity in as little as 15 seconds [73]. Considering
352 that Rhodnius adults are about 3 cm in length and dark colored, with a small thermal capacity, and
353 that they typically inhabit tropical environments with high humidity, they are likely to rapidly reach
354 temperatures above 60°C if exposed to full sun, suggesting that TRPA5 may mediate noxious heat
355 avoidance. Although the physiological and behavioral role of Rp-TRPA5$_2$ will need to be examined in
356 detail, the discovery of the activation range of TRPA5$_2$, opens avenues for exploring the convergent
357 evolution of noxious heat sensing between TRPA5$_2$ in Rhodnius bugs and *Pyrexia* in Drosophila flies,
358 and for assessing whether it is also relevant for both inner temperature regulation and warm substrate
359 avoidance.

360 To better understand the mechanisms of Rp-TRPA5$_2$ function and heat stimulus integration,
361 future studies could examine co-expression with other TRPs. Rp-TRPA5$_2$ is also expressed in
362 antennae, albeit at lower levels (Fig. S3). Antennae in triatomine bugs function as a multimodal
363 sensory organ, notably harboring highly specialized thermosensory sensilla involved in the detection
364 of host signals such as heat and moisture [36]. Heat sensing in triatomines is driven by air temperature
365 gradients around the host (conductive heat) [39, 40] as well as infrared radiation [74]. Antennal
366 thermoreceptors are known to be required for orientation and distance estimation [75] as well as for
367 precisely locating warm skin blood vessels before initiating proboscis extension and biting [76].
368 Similarly to homologous noxious heat detectors in mosquito antennae, Rp-TRPA5$_2$, might contribute
369 to tuning host-selective thermotaxis to avoid stimuli exceeding host temperatures. An interesting next
370 step would be to map the cellular location of Rp-TRPA5$_2$ in peripheral sensory neurons to investigate
371 potential colocalization with other channels involved in innocuous heat detection.

372 **TRPA5 as novel target to mitigate the impact *Rhodnius prolixus* in the transmission of Chagas**
373 **disease**

374 ThermoTRPs are polymodal sensors of physical and chemical stimuli [71]. For example, channels in
375 the insect TRPA1 and HsTRPA clades are typically activated by AITC and various plant-derived
376 chemicals such as carvacrol and citronellal [23, 28, 29]. However, characterized receptors of noxious
377 heat in insects such as Pyrexia and Painless do not exhibit chemical sensitivity to electrophiles [17].
378 Since our phylogenetic analyses support that Rp-TRPA5$_2$ is the sister clade to TRPA1, it is possible
379 that Rp-TRPA5$_2$ also plays a chemosensory role. Indeed, live Rhodnius treated with capsaicin, the
380 vanilloid pungent extract of chili peppers, were recently shown to have impaired orientation towards a
381 thermal source [77]. Notably, this compound can directly activate the mammalian TRPV1 receptor
382 independent of temperature, and the mammalian noxious temperature receptor, TRPV2, when bearing
383 only four mutations [3, 15]. Other than capsaicin, both TRPV1 and TRPV2 are readily activated by
384 additional vanilloid compounds such as resiniferatoxin, an active compound from the cactus *Euphorbia*
385 *resinifera* used for medicinal purposes and other plant-derived compounds that act as chemical
386 agonists [78]. Findings of botanical compounds triggering chemical activation of TRPA5$_2$ combined
387 with *in vivo* behavioral exposure studies, may thus contribute to uncovering new classes of natural
388 repellents potentially co-mediating heat-avoidance. This would be significant, not only in Rhodnius but
389 also for other triatomines and hemipteran vectors sharing a close TRPA5$_2$ orthologue such as the bed
390 bug, *Cimex lectularius*. Together, our study deorphanizes and characterizes the first ankyrin TRPA5
391 ion channel acting as noxious heat sensor, consistent with independent evolutionary origins of the
392 molecular transduction of noxious stimuli in insects, while simultaneously opening the door for further
393 pharmacological studies of TRP receptors in triatomine vectors.

394 **Material and Methods**

395 **Phylogenetic analyses.** Amino acid sequences of insect TRPA channels from the Anoplura (sometimes
396 included under Psocodea or Phthiraptera), Coleoptera, Diptera, Hemiptera, Hymenoptera, Isoptera and
397 Lepidoptera insect orders were retrieved from the InsectBase repository [79], FlyBase version FB2020_03 [80],
398 VectorBase (https://www.vectorbase.org), BeeBase [81], NCBI-blast [82], EnsemblMetazoa
399 (https://metazoa.ensembl.org), the i5k Workspace@ NAL [83] and OrthoFinder [84]. The TRP sequences from
400 insect model systems including *Drosophila melanogaster*, *Tribolium castaneum*, *Bombyx mori*, *Apis mellifera*
401 and *Rhodnius prolixus* were used as templates to mine and curate orthologous TRP ORF sequences from
402 annotated insect genomes and transcriptomes. To classify the uncharacterized TRPs, amino acid sequences
403 were aligned using MAFFT [85], and Maximum-Likelihood phylogenetic trees were inferred in IQ-TREE v1.6.11
404 using ModelFinder (Ultrafast Bootstrap, 1000 replicates), using a best-fit model JTT+F+I+G4 measured by the
405 Bayesian information criterion (BIC) [86-88]. The phylogenetic trees were visualized, rooted at mid-point and
406 annotated in R V3.6.3 using the ggtree package [89] and Evolview [90]. The accession numbers are listed in
407 Table S1.

408 **TRPA5 gene annotation and tissue expression**. We collected Illumina read data from *R. prolixus* tissue
409 libraries published in the Sequence Read Archive (SRA) at NCBI under Bioproject accession numbers
410 PRJNA281760/SRA:SRP057515 (antennal library from larvae, female adult and male adult),
411 PRJEB13049/SRA:ERP014587 (head library), and PRJNA191820/SRA:SRP006783 (ovary and testes library).
412 We performed low-quality base trimming and adaptor removal using cutadapt version 1.16 [91] and aligned the
413 trimmed read pairs against the *R. prolixus* assembly version RproC3.0.3 (retrieved from VectorBase.org)
414 genome using HISAT2 version 2.2.0 [92]. The existing annotation was used to create a list of known splice sites
415 using a python script distributed with HISAT2. We used StringTie version 2.1.3b [93] with the *conservative*
416 transcript assembly setting to improve the annotation, reconstruct a non-redundant set of transcripts observed
417 in any of the RNA-Seq samples, and compute expression estimates.

418 We applied Trinotate version 3.2.1 [94] to generate a functional annotation of the transcriptome data. In
419 particular, the functional annotation of TRP genes for which the initial genome annotation was absent or
420 incomplete (i.e *TRPA5, Nan, Pain*) were localized in Trinotate annotation followed by validation using the Apollo

421 gene browser [95]. Rp-TRPA5-A, Rp-TRPA5-B and Rp-TRPA5-C are three independent loci. Therefore, we
422 opted to rename them *Rp-TRPA5*$_1$, *Rp-TRPA5*$_2$, and *Rp-TRPA5*$_3$, respectively, to avoid confusion with standard
423 gene isoform nomenclature, which applies in the case of TRPA1-A and TRPA1-B, two isoforms of the Rhodnius
424 *TRPA1* locus. All TRP gene identifiers are presented in Table S2.

425 The alignment BAM files were used to estimate transcript abundance using StringTie together with our improved
426 annotation. The abundance tables from StringTie were imported into R using the *tximport* package [96], which
427 was used to compute gene-level abundance estimates reported as FPKM. We used the R package *pheatmap*
428 to visualize the expression level of TRP genes.

429 **Monitoring of TRPA5$_2$ expression levels by quantitative PCR.** Live adults of *R. prolixus* were obtained from
430 BEI Resources (USA). Female antennae, rostrum, legs, heads (minus antenna and rostrum), and bodies (thorax
431 minus legs + abdomen) were dissected and pooled from 15 individuals in DNA/RNA shield reagent (Zymo) and
432 stored at -20°C until further processing. Total RNA was isolated using the Monarch RNA extraction procedure
433 (New England Biolabs), including tissue grinding in liquid nitrogen and a DNAse I step. cDNAs were synthesized
434 using the GoScript cDNA synthesis procedure (Promega) prior to concentration assessment using the Qubit
435 High sensitivity DNA kit (Invitrogen). Two gene-specific primer (GSP) sets were designed for Rhodnius Actin
436 (Genbank acc. Nr. EU233794.1) and TRPA5$_2$ using Primer3 version 2.3.7 in Geneious [97] (Table S3). Each
437 primer set was initially validated by calculating standard curves from serial dilutions of template cDNA (2 ng/µL
438 to 0.25 ng/µL) and primer mix (5 to 0.25 µM) with choosing amplification efficiencies (E) between 95 and 100%.
439 qPCR amplification products from initial runs were additionally checked on 2% agarose gels to verify the correct
440 amplicon sizes and the absence of primer dimers. As a final validation, qPCR products were purified using Exo-
441 SAP (Fermentas) prior to Sanger sequencing to ensure product amplification specificity. Quantitative PCR
442 reactions were then run in three technical replicates on a CFX384 Real-Time PCR system (Bio-Rad) with
443 quantification and dissociation curves analyses performed for three independent experiments using the CFX
444 Maestro Software 2.3 (Bio-Rad). Each five-microliter reaction contained 2.5 µL 2x SsoAdvanced Universal SYBR
445 Green Supermix (Biorad), 0.25 ng cDNA and 0.125 µM primers. Cycling conditions were as follows: 95°C for 2
446 min, 39 cycles of 95°C for 10 s, 60°C for 10 s followed by a dissociation curve analysis from 65.5°C to 89.5°C
447 with gradual heating at 0.6°C/s. Relative log-fold expression levels were normalized per tissue type against the
448 reference gene and calibrated relative to Antennae (log fold expression = 1).

449 **Alpha-fold modeling and DALI analyses.** Monomer structures of Rhodnius TRPA1, Rhodnius Painless,
450 Rhodnius Waterwitch, Rhodnius TRPA5$_2$, Drosophila TRPA1, Drosophila Painless, Drosophila Waterwitch and
451 Drosophila Pyrexia were generated using AlphaFold2 with amber relaxation activated [51] on Colab's server
452 [53]. To model the Rhodnius TRPA5$_2$ tetramer, due to limitations in computational power, the transmembrane
453 region (residues 608-1078) was modeled first, and then used as a custom template to model a monomer of
454 residues 42-1078. The first 41 residues and the C-terminal of the monomers from residue 1079 were disordered
455 and truncated to avoid clashes when assembling the tetramer. A tetramer was assembled of four copies of the
456 monomer by aligning them to each of the chains of the truncated transmembrane tetramer in PyMOL [98]. The
457 monomer models were compared with pairwise structural alignment using the Dali server [99]. The PDB files
458 are provided as supplementary material.

459 **Molecular cloning.** Antennae from twenty Rhodnius adult individuals were obtained from a laboratory culture
460 (Orchard lab, University of Toronto Mississauga, Canada) and stored in DNA/RNA Shield™ reagent (Zymo
461 Research). Tissues were disrupted in Trizol using a Premium Multi-Gen 7XL Homogenizer (PRO Scientific) and
462 RNA was subsequently extracted using the Direct-zol RNA kit (Zymo Research), including a DNAse step to
463 remove genomic DNA contamination. cDNA was synthesized from 1ug Total RNA using the GoScript™ Reverse
464 Transcriptase kit (Promega) and random hexamers following the recommended manufacturers' protocol. RNA
465 and cDNA qualities were verified using a Nanodrop (Nanodrop 2000/2000c UV-vis spectrophotometer, Thermo
466 Scientific) and quantified using a Qubit Fluorometer (ThermoFisher). The coding regions of Rhodnius *Rp-*
467 *TRPA5$_2$* was amplified from antennal cDNA using gene-specific primers designed based on Rhodnius full length
468 TRP sequences [37] and containing unique restriction sites (Table S3). PCR reactions were performed in a
469 Veriti™ Thermal Cycler (ThermoFisher) using the Advantage® 2 PCR Kit (Takara Bio) in a touchdown cycling
470 program as follows: 95°C for 2 min, 16 cycles of 95°C for 30 sec, 68°C for 1 min (-0.5°C/cycle), 68°C for 4 min
471 followed by 20 cycles of 95°C for 30 sec, 60°C for 1 min, 68°C for 4 min, and a final step at 68°C for 10 min.
472 Amplification products were analyzed by electrophoresis, and fragments of expected size were excised from the
473 gel, purified using the Monarch® DNA gel extraction kit (NEB) and subjected to Sanger Sequencing for ORF
474 sequence-verification prior to codon-optimization at Genscript and subcloning. For the rat rTRPV1 and the fruit
475 fly Dm-TRPA1-D, gene specific primers (Table S3) were used to amplify the ORF including suitable flanking
476 restriction sites prior to gel purification and double restriction digestion. The digested PCR products were gel

477 purified and ligated in an expression cassette containing the human cytomegalovirus (CMV) immediate early
478 promoter and engineered to include a C-terminal tag by the monoclonal antibody FLAG epitope sequence
479 (DYKDDDDK), followed by a Ser-Gly-Ser linker peptide, a T2A peptide sequence (EGRGSLLTCGDVEENPG)
480 and the coding region of the cytoplasmic fluorescent marker protein mRuby2 [62, 100]. The ligation mixtures
481 were used to transform *Stbl3* competent *E. coli* cells (ThermoFisher) using standard protocols. Plasmid DNAs
482 were purified using the Qiaprep spin Miniprep (Qiagen) and verified by Sanger sequencing using internal gene-
483 specific and vector primers to ensure overlapping sequence information in both forward and reverse directions.
484 High yield pure plasmid DNA preparations were subsequently obtained from 100 mL overnight LB broth cultures
485 using the endo-free ZymoPURE™ II Plasmid Midiprep Kit (Zymo Research, USA).

486 **Transient HEK293T cell expression.** Plasmid DNAs clones from TRP cDNAs were transiently expressed in
487 HEK293T cells to optimize expression conditions via mRuby2 visualization and western blot analysis prior to
488 whole cell patch clamp recordings. HEK293T cells were seeded at a density of 0.6 x 10^6 cells on day 0 in 60 mm
489 culture dishes (ref 25382-100, VWR) in DMEM High Glucose, GlutaMAX (Life Technologies) supplemented with
490 10% FBS (Seradigm Premium, VWR, USA). For each transfection, lipid complexes containing 2.5 µg DNA: 10
491 µL L2000 (Life Technologies) mixed in Opti-MEM I Reduced Serum (Life Technologies) were added dropwise
492 to the cells at 50% confluency (1.2 x 10^6 cells, day 1). The culture medium was exchanged with new DMEM/FBS
493 medium six-hours post-transfection. Cells were incubated at 37°C in a humidified HERAcell 150i incubator
494 (Thermo Scientific) with 5% CO_2.

495 **Biochemistry.** For whole-cell TRP expression analysis, cells were harvested 72h post-transfection; the medium
496 was decanted, cells were collected in 2mL cold D-PBS, centrifuged for 5 min at 4,000 rpm at 4°C and then the
497 supernatant was discarded. The cell pellet was gently suspended in 50 µL cold Ripa lysis buffer (Thermo
498 Scientific) supplemented with 1% Triton-X100 (Sigma-Aldrich) and complete EDTA-free protein inhibitors
499 (Sigma-Aldrich). Cell membranes were lysed for 1h at 4°C with gentle rotation on a sample homogenizer, and
500 cell debris were collected by centrifugation at 4°C for 15 min at 13,000 rpm. The crude protein lysate
501 concentration was quantified by bovine serum albumin (BSA) (Sigma-Aldrich) and 25 µg crude extract was
502 loaded on NuPAGE™ 3-8% Tris-Acetate gels (ThermoFisher) and transferred to a polyvinylidene difluoride
503 membrane on a TurboBlotTransfer system (Bio-Rad Laboratories). The membranes were blocked with 5% milk
504 (Bio-Rad) in Tris-buffered saline containing 0.1% Tween 20 (TBST, Bio-Rad) and incubated overnight with
505 aFLAG antibody 1:2,500 (GE Healthcare) on a gently rocking platform at 4°C. After washing with TBST the

506 membranes were incubated for 1h at ambient temperature in the dark with horseradish peroxidase (HRP) ECL
507 anti-mouse conjugated antibody (Amersham, USA) diluted in 5% milk in TBS-Tween at 1:2,500. Membranes
508 were rinsed in TBST and revealed using the SuperSignal West Femto (Thermo Scientific) and imaged on a
509 ChemiDoc system (Bio-Rad Laboratories).

510 For membrane surface expression, the plasma membrane expression of Rp-TRPA5$_2$ channels was assessed
511 using the Pierce Cell surface Protein isolation kit (Thermo Scientific). On day 0, four T75 cm^2 flasks were seeded
512 with 1 x 10^6 HEK293T cells. Forty hours later, each flask was transfected with lipid complexes containing 48 µg
513 endo-free plasmid DNA and 96 µl Lipofectamine 2000 diluted in Opti-MEM serum and incubated at 37°C. 72
514 hours post-transfection, cells were gently washed with ice-cold PBS, labeled with Sulfo-NHS-SS-Biotin, and
515 harvested following the manufacturer's protocol. Cells were lysed on ice for 30 min in the manufacturer's lysis
516 buffer supplemented with 0.5% Triton-X100 and complete EDTA-free protein inhibitors (Sigma-Aldrich), with
517 gentle 5s vortexing every 5 min, and two 5x-1s sonicating bursts on ice. Following centrifugation, the cell lysate
518 was bound to NeutrAvidin agarose resin and gently mixed for 60 min at ambient temperature on a platform
519 rotator. The membrane-bound fraction was eluted with 50mM Dithiothreitol in SDS-Sample buffer (62.5 mM
520 Tris/HCl pH6.8, 1% SDS, 10% Glycerol) and then placed on ice. For Western Blot analysis, 32 µl of the
521 membrane protein eluate fraction were mixed with Laemmli buffer (Bio-Rad) supplemented with 10% 2-
522 mercaptoethanol. Sixteen µl of the homogenized protein-loading buffer sample were loaded in duplicates on a
523 NuPAGE™ 3-8% Tris-Acetate gel (ThermoFisher) to be probed separately with FLAG and ATPase antibodies.
524 Proteins were separated by electrophoresis for 3h at 80V at 4°C, then transferred to a polyvinylidene difluoride
525 membrane on a TurboBlotTransfer system (Bio-Rad Laboratories). The membranes were blocked in parallel
526 with 5% milk (Bio-Rad) in Tris-buffered saline containing 0.1% Tween 20 (TBS-T, Bio-Rad) and incubated
527 overnight on a gently rocking platform at 4°C with aFLAG antibody 1:2,500 (GE Healthcare) or with Anti-Sodium
528 Potassium ATPase antibody 1:2,500 (ab76020, Abcam) diluted in 5% milk. After three washes with TBST, the
529 membranes were incubated for 1h at ambient temperature in the dark with HRP ECL anti-mouse conjugated
530 antibody (Amersham, USA) at a 1:2,500 dilution in 5% milk/TBST. Membranes were rinsed in TBST and revealed
531 using the SuperSignal West Femto (Thermo Scientific) and imaged on a ChemiDoc system (Bio-Rad
532 Laboratories).

533 **Temperature control using a laser system**. We used a manual patch-clamp station (Axopatch 200, Molecular
534 Devices) equipped with a fiber-delivered laser system to record temperature-activated currents under a precise
535 voltage-clamp control. The setup was modified after Yao et al (2009) [101] (Fig. S5) and takes advantage of
536 water's IR absorption band to generate rapid temperature jumps from RT to high temperatures. It combines an
537 infrared diode laser (λc =1460 nm (+/-20 nm), Output power = 4.8 watts) (Seminex Inc.) coupled with a 100-um
538 optical fiber with a striped tip (ThorLabs, Inc.) as the controllable heat source. Two independent
539 micromanipulators allowed us to precisely align the relative positions of the patch-clamp electrode and the fiber
540 on a single cell (Fig. S8). To calibrate the optic fiber position with respect to the patch pipette we used a visible
541 laser (Fig. S8). Marks on the computer screen were used to keep the position of the fiber and the pipette
542 consistent for the different experiments. Cells under whole-cell voltage-clamp control were held at -30mV during
543 the experiment. To program fast pseudo-transient temperature changes, the patch pipette current was used to
544 read the temperature changes in real-time as the feedback to the laser diode controller (LDC-37620, ILX
545 Lightwave) to perform proportional-integral-derivative (PID) control of the driving current of the laser diode (*see*
546 *Extended Methods*). This laser-heating setup provides a rapid and precise heating rate on the order of 50°C
547 within tens of milliseconds, essential to provide both adequate temporal resolution and controllable steady-state
548 temperatures in the range of 35°C to 70°C to analyze the channel activation. Fig. S7A shows constant
549 temperature steps were achieved with a rising time constant of 34.2±3.3 ms, independent of the laser power.
550 The temperature jump associated with successive current pulses is precisely calculated by running an open
551 pipette calibration following the same current sequence at the end of each run (Fig. S6).

552 **Temperature calibration.** We used the resistance of the open pipette to measure the temperature jump
553 magnitudes following the equation $T=\{1/T_0 - R/E_a \times \ln(I/I_0)\} - 1$, where R is the gas constant, T_0 and I_0 are
554 respectively room temperature and the corresponding electrode current at room temperature. The activation
555 energy (E_a) of the system corresponds to 3.84 kcal/mol as was established by Yao et al. (2009) for the pair of
556 solutions used in the recordings [101]. The equation describes the change in ion motility as a function of
557 temperature changes in the system. The current change was used as a feedback signal for a laser-diode
558 controller software coded in Labview that uses a proportional-integral-derivative (PID) control algorithm. To
559 account for the variability in the diameter between the different patch pipettes used in different experiments, the
560 instrument was calibrated before each experiment to assure comparable temperature jumps in each experiment,
561 adjusting the diode power outputs to the desired temperature accordingly.

562 **Whole cell patch-clamp recordings.** Cells were seeded at low density in a 30 mm culture dish (VWR)
563 containing round glass coverslips 48h post-transfection (Table S5, Fig. S5). Cells were first rinsed with D-PBS
564 at room temperature, trypsinized with 0.5 mL Accutase (Stemcell Technologies) and suspended in 4.5 mL pre-
565 warmed DMEM-FBS medium. Two hundred microliters of this cell suspension were mixed with 1.8 mL pre-
566 warmed DMEM-FBS medium, dispensed drop wise in the culture dish, and incubated for 24h at 30°C. In a typical
567 experiment, one glass cover slip was gently retrieved from the culture dish using sterile forceps, rinsed with a
568 recording solution using a Pasteur pipette and placed in the recording chamber (Fig. S6). The fluorescence of
569 mRuby-expressing cells was monitored to select bright, healthy, isolated cells for whole-cell patch clamping.
570 Experiments under the whole-cell configuration were carried out 72h after induction. The electrodes were
571 fabricated with borosilicate capillaries, using a horizontal micropipette puller P-1000 (Sutter Instrument, Novato,
572 CA, USA), and polished using a microforge (Narishige, Japan) to a final diameter between 2-4 um. The internal
573 electrode was filled with the following solution in mM: 126 CsCl, 10 EGTA, 10 HEPES, 1 EDTA, and 4 MgATP.
574 The extracellular recording solution contained 2 mM $CaCl_2$, 10 mM HEPES, 140 mM NaCl, pH 7.2 (adjusted with
575 NaOH). The electrode resistance ranged between 2-4 MΩ, and the Vjp was estimated at ~18 mV for the
576 recordings. The current traces were amplified using a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale,
577 CA, USA). The amplified electrical signals were acquired with a Digidata 1440A using the pClamp10 software
578 (Molecular Devices, Sunnyvale, CA, USA). Series Resistance (Rs) was compensated in 80%, as well as the fast
579 and slow capacitive components of the current. The current density was fitted to the following Boltzmann
580 function:

581 $$I_total = I_leak\ e^{(-(\Delta H_leak)/RT)} + (I_max\ e^{(-(\Delta H_i)/RT))}/(1 + e^{(-(\Delta G_T)/RT)})$$

582 Whereby the first term ΔH_leak is the enthalpy change of the leak current. The second term accounts for the
583 channel activity, with $\Delta G = \Delta H - T\Delta S$ is the free energy change involved in the closed-open reaction, and ΔH_i
584 accounts for the linear temperature dependence of the ionic conductivity and leakage current [101]. The
585 corrected temperature current density (*I*) was used to calculate the equilibrium constant from the relative fraction
586 of the channel in the open conformation (Po), assuming a two-state model, where Po = I/Imax.

587 $$Po = 1/(1 + Keq^{-1})$$

588 where $\ln(K_{eq}) = -(\Delta H/RT) + (\Delta S/R)$. Thus from the Van't Hoff plots ln(Keq) vs 1/T, the enthalpy and entropy
589 associated with the channel opening can be obtained [66].

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603 **Data Availability Statement**

604 All datasets generated and analyzed for this study will be published as supplementary files
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870

871 **Extended data**

Convergent evolution of noxious heat sensing by TRPA5, a novel class of heat sensor in *Rhodnius prolixus*

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882 **This document contains:**

883 **Supplementary Methods**

884 **Supplementary Figures S1 to S8**

885 **SI Tables S1 to S5 are in a separate file**

886 **Supplementary Methods**

887 **PID control**

888 A PID (proportional-integral-derivative) control is the typical way to adjust the output according to the input
889 reading in real-time without knowing most of the environmental parameters. The idea is described in the following
890 function:

891 $$Output(t) = K_p Err(t) + K_i \int_0^t Err(\tau) d\tau + K_d \frac{dErr(t)}{dt}$$

892 $$Err(t) = Input(t) - Setpoint$$

893

894 with $Output(t)$ being the laser power, $Input(t)$ being the temperature, and $Setpoint$ being the desired value.
895 The $Output(t)$ is determined by $Err(t)$, which is the difference between $Input(t)$ and $Setpoint$.

896 There are 3 terms in this equation, the first term is the proportional term. This term varies linearly with $Err(t)$.
897 For instance, when the temperature reaches the setpoint, this term decreases, and when the temperature
898 exceeds the setpoint, this term becomes negative to bring the temperature back to the setpoint.

899 The second term is the integral term. This term provides a gradually increasing offset and this offset will stabilize
900 when the temperature stabilizes to setpoint, where $Err(t) \to 0$.

901 The third term is the derivative term. This term estimates the required change of output by watching the inertia
902 of $Err(t)$. For instance, when the temperature reaches the setpoint, the proportional term gives 0 and the integral
903 term gives a stabilized value, but if the temperature is still increasing, this term will decrease the output to prevent
904 the temperature from exceeding the setpoint in the next time interval.

905

906 **Open pipette current measurements**

907 The temperature is measured by monitoring the current through an open patch pipette, which means there are
908 no cells but only water, assuming the thermal property of the cell is the same as water. First, a series of open-
909 pipette measurements with different laser powers and power waveforms is performed and used to calculate the

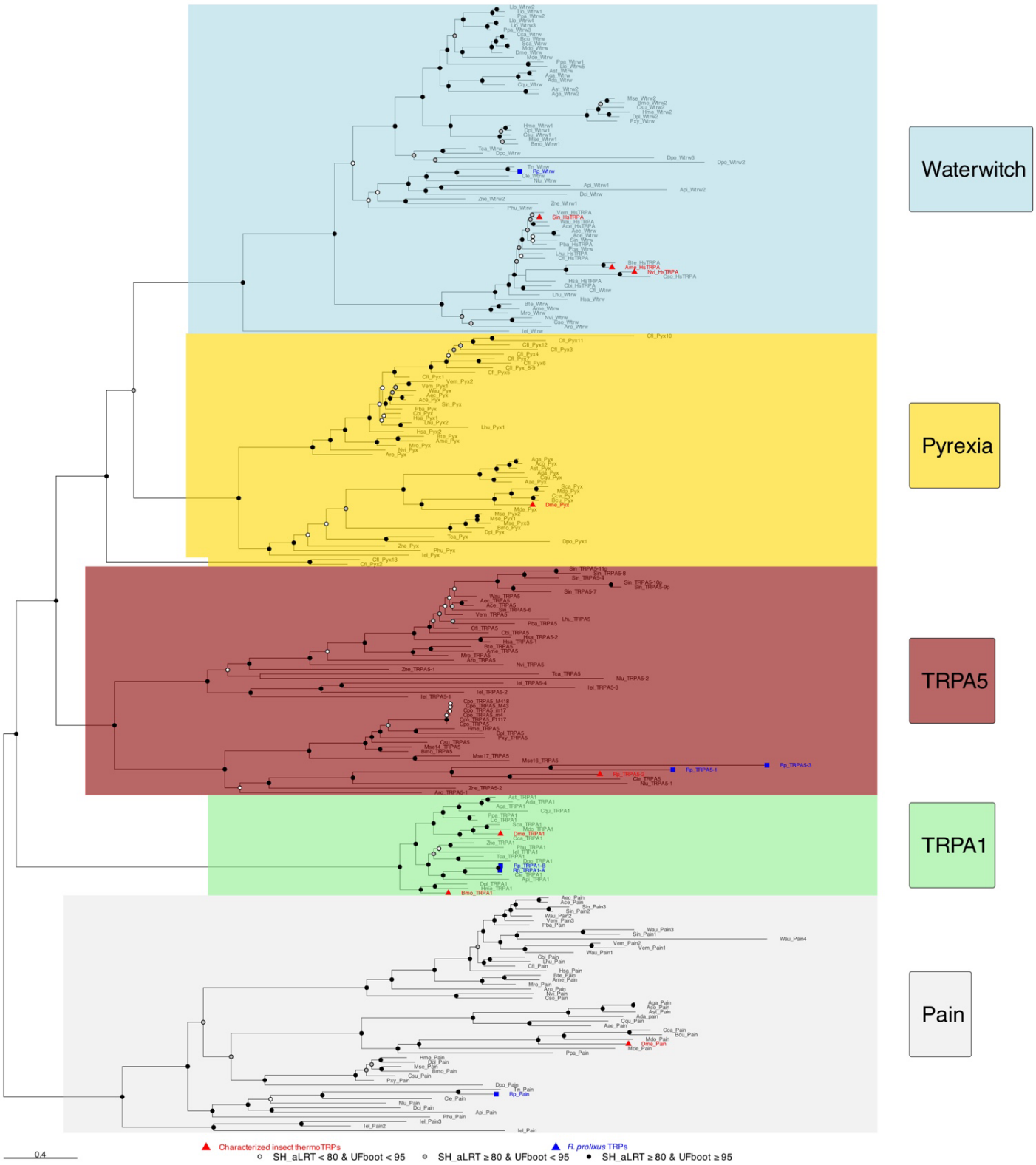
910 temperature evolution from real-time current in the patch-clamp recording pipette. The patch-clamp experiment
911 is conducted by applying the same laser powers and waveforms to the cell (Fig. S4)

912 **Fiber preparation**

913 The attenuation coefficient of water at the wavelength of 1460 nm is ~3000 cm^(−1) which means the absorption
914 of the laser power through a 100nm thick water layer is over 40%. Using the fiber above the water surface, the
915 laser would thus deliver most of its power in the upper water layer. The temperature of water above the targeted
916 cell would be much higher than the cell temperature itself, and the temperature change would not be confined
917 to a single cell due to conduction and convection in the water. To resolve this, we cut the fiber with a diamond
918 blade under a stereomicroscope and stripped the fiber which allowed us to place the fiber tip in the water layer
919 directly above the cell in the recording chamber (Fig. S5). The relative position of the fiber tip and cell was
920 adjusted using patch-clamp rig microscope.

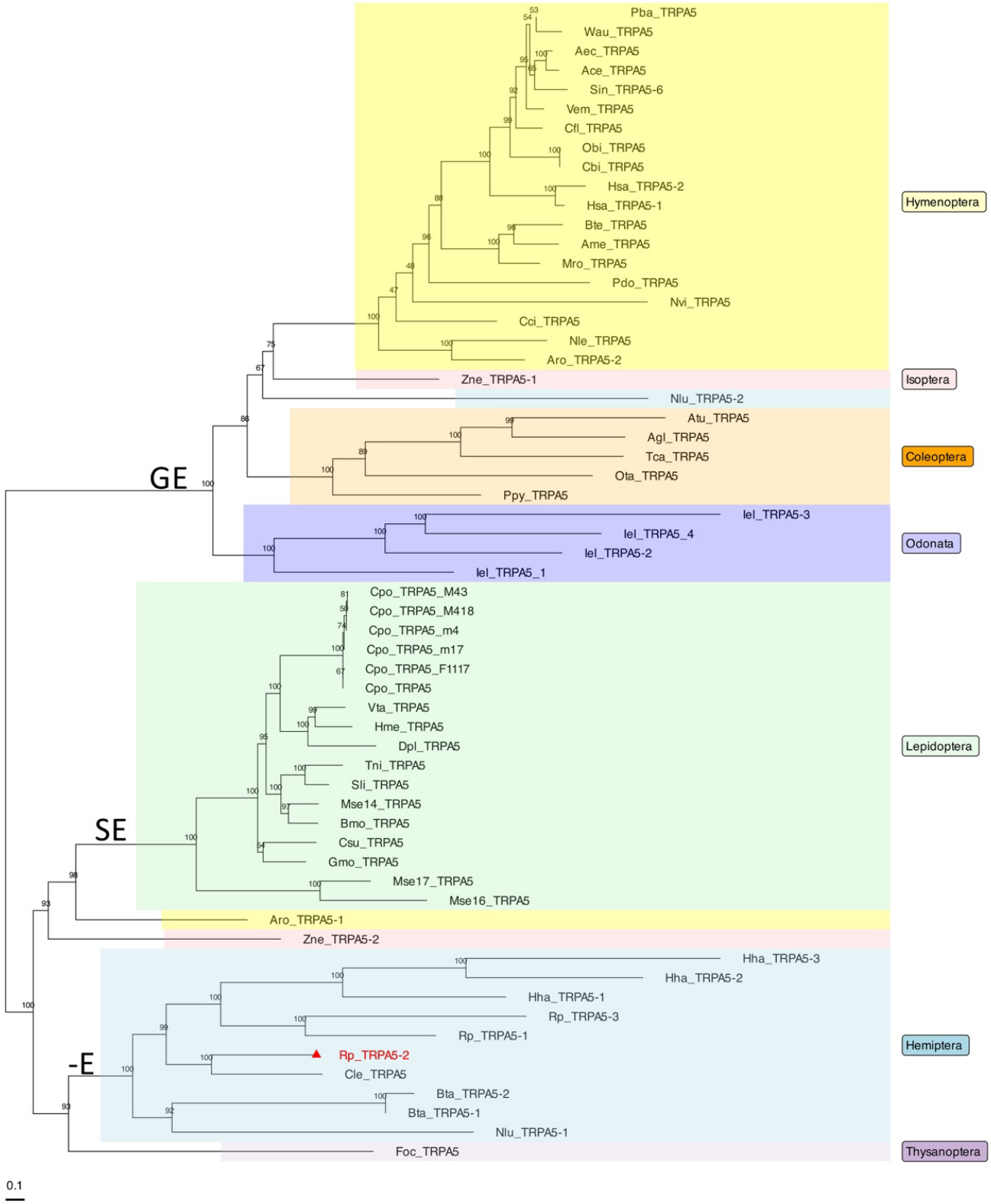
Supplementary Figures

Figure S1. Phylogeny of insect TRPA channels



923 **Figure S1. Phylogeny of insect TRPA channels.** The Maximum-likelihood phylogeny of amino acid sequences
924 includes representative ion channel members of the TRPA1, Pain, Pyrexia, HsTRPA, TRPA5, Wtw subfamilies.
925 Accession numbers are listed in Table S1. The tree was inferred in IQ-TREE v1.6.11 using ModelFinder,
926 Ultrafast Bootstrap (UFboot), 1000 replicates, using a best-fit model JTT+F+I+G4 measured by the Bayesian
927 information criterion (BIC). Branches were assigned Shimodaira-Hasegawa approximate likelihood ratio test
928 (SH-aLRT) and UFboot supports. The tree was visualized in Rstudio (2021.09.2) using ggtree (Script provided).
929 Species represented are **Anoplura**: *Pediculus humanus* (Phu, head louse), **Coleoptera**: *Tribolium castaneum*
930 (Tca, red flour beetle), *Dendroctonus ponderosae* (Dpo, Mountain pine beetle), *Photynus pyralis* (Ppy, Common
931 Eastern Firefly), *Agrilus planipennis* (Apl, Emerald ash borer), *Anoplophora glabripennis* (All, Asian longhorned
932 beetle), *Aethina tumida* (Aut, Small hive beetle), *Onthophagus taurus* (Ota, bull dung beetle), **Diptera**:
933 *Drosophila melanogaster* (Dme, fruitfly), *Mayetiola destructor* (Mde, Hessian fly), *Culex quinquefasciatus* (Cqu,
934 Southern house mosquito), *Anopheles stephensi* (Ast), *Anopheles gambiae* (Aga), *Anopheles darlingi* (Ada),
935 *Bactrocera cucurbitae* (Bcu, Melon fly), *Ceratitis capitata* (Cca, Mediterranean fruit fly), *Musca domestica* (Mdo,
936 housefly), *Lutzomyia longipalpis (Llo, Sandfly), Phlebotomus papatasi* (Ppa, Sandfly), *Stomoxys calcitrans* (Sca,
937 Barn fly), **Hemiptera:** *Acyrthosiphon pisum* (Api, pea aphid), *Nilaparvata lugens* (Nlu, Brown planthopper),
938 *Diaphorina citri* (Dci, Asiatic citrus psyllid), *Cimex lectularius* (Cle, Bed bug), *Triatoma infestans* (Tin, Winchuka)
939 *Rhodnius prolixus* (Rp, Kissing bug), **Hymenoptera:** *Apis mellifera* (Ame, Western honeybee) , *Bombus*
940 *terrestris* (Bte, Buff-tailed bumblebee), *Megachile rotundata* (Mro, Alfalfa leaf cutting bee), *Nasonia vitripenis*
941 (Nvi, Jewel wasp), *Harpegnathos saltator* (Hsa, Jumping ant), *Linepithema humile* (Lhu, Argentine ant),
942 *Campanatus floridanus* (Cfl, Florida carpenter ant), *Pogonomyrmex barbatus* (Pba, red harvester ant), *Atta*
943 *cephalotes* (Ace, Leafcutter ant), *Acromyrmex echinatior* (Aec, Panamian leafctutter ant), *Solenopsis invicta*
944 (Sin, Red imported fire ant), *Vollenhovia emeryi* (Vem, ant), *Athalia rosae* (Aro, Turnip sawfly), *Cerapachys biroi*
945 (Cbi, Clonal raider ant), *Ceratosolen solmsi* (Cso, Fig wasp), *Wasmannia auropunctata* (Wau, Electric ant),
946 **Isoptera:** *Zootermopsis nevadensis* (Zne, Dampwood termite), **Lepidoptera**: *Bombyx mori* (Bmo, Silkmoth),
947 *Chilo suppressalis* (Csu, Asiatic rice borer), *Danaus plexippus* (Dpl, Monarch butterfly), *Heliconius melpomene*
948 (Hme, Postman butterfly), *Plutella xylostella* (Diamondback moth), *Manduca sexta* (Mse, Tobacco hornworm)
949 *Cydia pomonella* (Cpo, Codling moth), **Odonata**: *Ischnura elegans* (Iel, Bluetail damselfly).

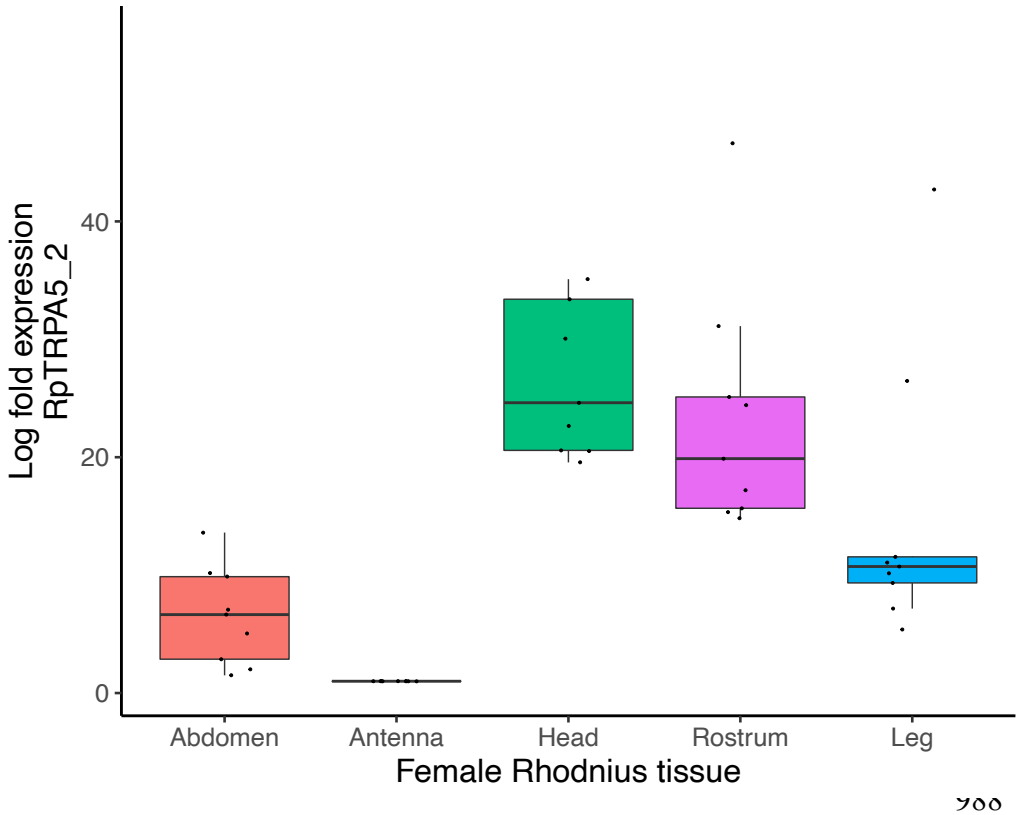
950 **Figure S2. Phylogeny of insect TRPA5 channels**



951

952 **Figure S2. Phylogeny of insect TRPA5 channels.** TRPA5 channels are present across insect Orders but
953 Diptera. The Maximum-likelihood phylogeny of amino acid sequences includes representative ion channel
954 members of the TRPA5 subfamily. The tree was inferred in IQ-TREE v1.6.11 using ModelFinder, Ultrafast
955 Bootstrap (UFboot), 1000 replicates, using a best-fit model JTT+F+I+G4 measured by the Bayesian information
956 criterion (BIC). Branches were assigned Shimodaira-Hasegawa approximate likelihood ratio test (SH-aLRT) and
957 UFboot supports. The tree was visualized in Rstudio (2021.09.2) using ggtree (script provided). Species
958 abbreviations are as follows: **Coleoptera:** *Tribolium castaneum* (Tca, red flour beetle), *Photynus pyralis* (Ppy,
959 Common Eastern Firefly), *Anoplophora glabripennis* (Agl, Asian longhorned beetle), *Aethina tumida* (Aut, Small
960 hive beetle), *Onthophagus taurus* (Ota, bull dung beetle); **Hemiptera**: *Bemisia tabaci* (Bta, Silverleaf Whitefly),
961 *Cimex lectularius* (Cle, Bed bug), *Halyomorpha halys* (Hha, Brown marmorated stinkbug), *Nilaparvata lugens*
962 (Nlu, Brown planthopper), *Rhodnius prolixus* (Rp, Kissing bug); **Hymenoptera**: *Apis mellifera* (Ame, Western
963 honeybee) , *Athalia rosae* (Aro, Turnip sawfly), *Bombus terrestris* (Bte, Buff-tailed bumblebee), *Megachile*
964 *rotundata* (Mro, Alfalfa leaf cutting bee), *Nasonia vitripennis* (Nvi, Jewel wasp), *Harpegnathos saltator* (Hsa,
965 Jumping ant), *Linepithema humile* (Lhu, Argentine ant), *Campanatus floridanus* (Cfl, Florida carpenter ant),
966 *Pogonomyrmex barbatus* (Pba, red harvester ant), *Atta cephalotes* (Ace, Leafcutter ant), *Acromyrmex echinatior*
967 (Aec, Panamian leafcutter ant), *Solenopsis invicta* (Sin, Red imported fire ant), *Vollenhovia emery* (Vem, ant),
968 *Ooceraea biroi* (Cbi, Clonal raider ant), *Wasmannia auropunctata* (Wau, Electric ant), *Polisted dominula* (Pdo,
969 European paper wasp), *Neodiprion lecontei* (Nle, Red-headed pine sawfly), *Cephus cinctus* (Cci, Wheat stem
970 sawfly); **Isoptera**: *Zootermopsis nevadensis* (Zne, Dampwood termite); **Lepidoptera:** *Bombyx mori* (Bmo,
971 Silkmoth), *Chilo suppressalis* (Csu, Asiatic rice borer), *Danaus plexippus* (Dpl, Monarch butterfly), *Heliconius*
972 *melpomene* (Hme, Postman butterfly), *Plutella xylostella* (Pxy, Diamondback moth), *Manduca sexta* (Mse,
973 Tobacco hornworm), *Cydia pomonella* (Cpo, Codling moth), *Vanessa tameamea* (Vta, Kamehameha butterfly),
974 *Trichoplusia ni* (Tni, Cabbage looper), *Spodoptera litura* (Sli, Tobacco cutworm), *Galleria mellonella* (Gmo,
975 Greater wax moth), **Odonata**: *Ischnura elegans* (Iel, damselfly), **Thysanoptera**: *Frankliniella occidentalis* (Foc,
976 thrips). Capital letters above branches represent the amino acid residues at the ion channel selectivity filter (see
977 Fig. 2).

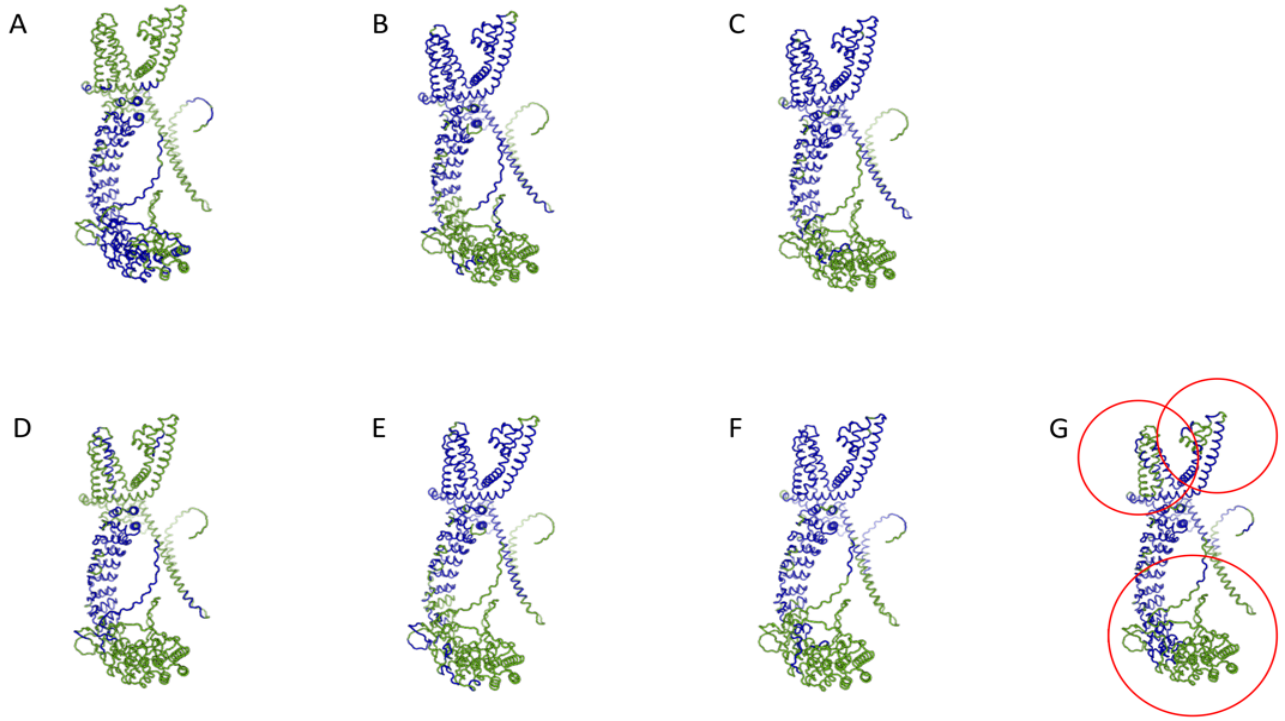
978 **Figure S3. qCPR analysis**



988

989 **Figure S3.** Differential expression pattern of Rp-TRPA5$_2$ transcripts isolated from *R. prolixus* female sensory
990 tissues and monitored by quantitative PCR. The mean relative expression scores were calculated from raw cycle
991 threshold (Ct) values (±SEM, n= 9) (Table S5) and boxplots were visualized in R (script provided). Log$_2$ fold
992 change expression values are shown relative to the *Rhodnius* housekeeping gene (Actin) and calibrated to
993 expression in Antenna.

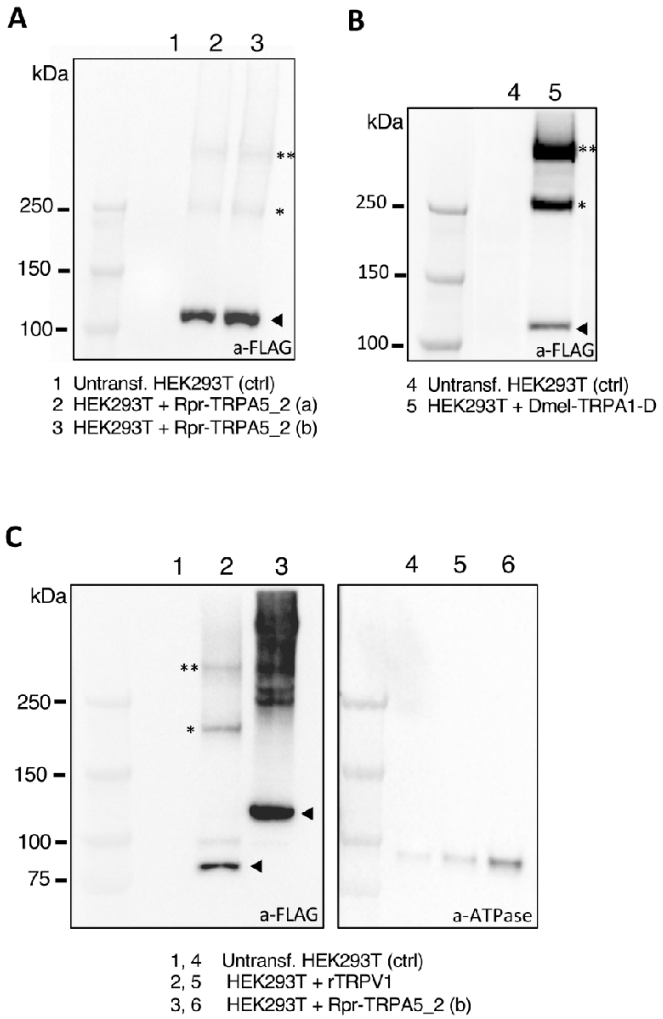
994 **Fig. S4. DALI Analysis**



995

996 **Figure S4. DALI Analysis.** Pairwise structural alignment using the Dali server
997 (http://ekhidna2.biocenter.helsinki.fi/dali/), of different monomers against Rhodnius TRPA5$_2$. **A.** Rhodnius
998 TRPA1, **B**. Rhodnius Painless, **C.** Rhodnius Waterwitch, **D.** Drosophila TRPA1, **E.** Drosophila Painless, **F.**
999 Drosophila Waterwitch, **G.** Drosophila Pyrexia. The red circles in **G** indicate areas where the Pyrexia monomer
1000 stands out in that the pore region and the voltage sensor domain are less similar to TRPA5$_2$, but the ARD is
1001 more similar to TRPA5$_2$.

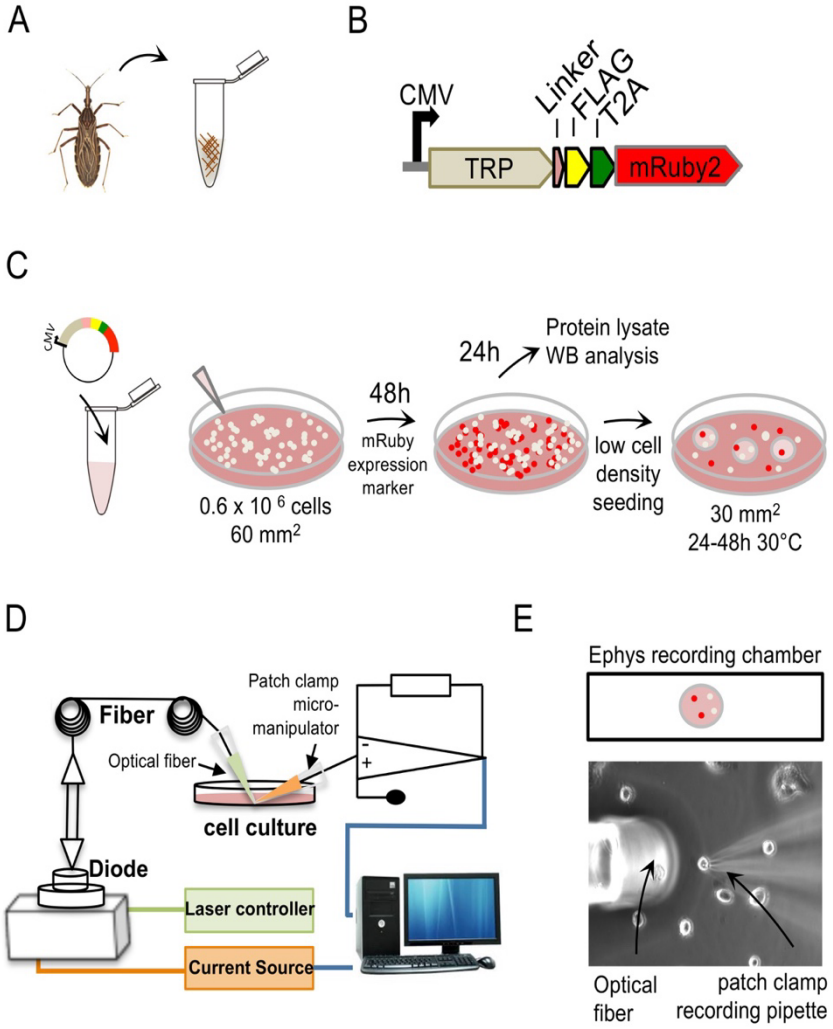
1002 **Figure S5. TRP whole cell and surface expression.**



1003 **Figure S5. A-B**. **Rp-TRPA5$_2$ anti-FLAG signals in whole cell lysates**. Untransfected HEK293T cells (lanes
1004 1,4) and HEK293T cells transfected with 2.5 µg pcDNA-FLAG-T2A-mRuby plasmid DNAs for Rp-TRPA5$_2$ (A,
1005 lanes 2,3), and fruitly Dmel-TRPA1-D (B, lane 5). Cells were collected 72 hrs after transfection. The protein
1006 ladder image taken from the same membrane is juxtaposed to the left of the immunoblot. One and two asterisks
1007 represent predicted dimeric and tetrameric TRP forms, respectively. The predicted monomeric MW is indicated
1008 with a black arrowhead: Rp-TRPA5$_2$, 127.78 kDa; fruit fly Dmel-TRPA1-D 138.82 kDa. **C.** Surface expression
1009 analysis of Rp-TRPA5$_2$. Biotinylated surface protein eluates were run in parallel wells on the same SDS-page
1010 gel to probe TRP (left) and ATPase (right). Anti-FLAG levels in surface protein fraction are from non-transfected
1011 HEK293T cells (lane 1), cells expressing rTRPV1 (lane 2), and cells expressing Rp-TRPA5$_2$ (lane 3). Lanes 4
1012 to 6 are the corresponding anti-ATPase biotin-surface fraction from non-transfected HEK293T cells (lane 4),
1013 cells expressing rTRPV1 (lane 5), and cells expressing Rp-TRPA5$_2$ (lane 6). One and two asterisks represent
1014 predicted dimeric and tetrameric TRP forms, respectively. The predicted monomeric MW is indicated with a black
1015 arrowhead (rTRPV1, 94.95 kDa; Rp-TRPA5$_2$, 127.78 kDa).

1016

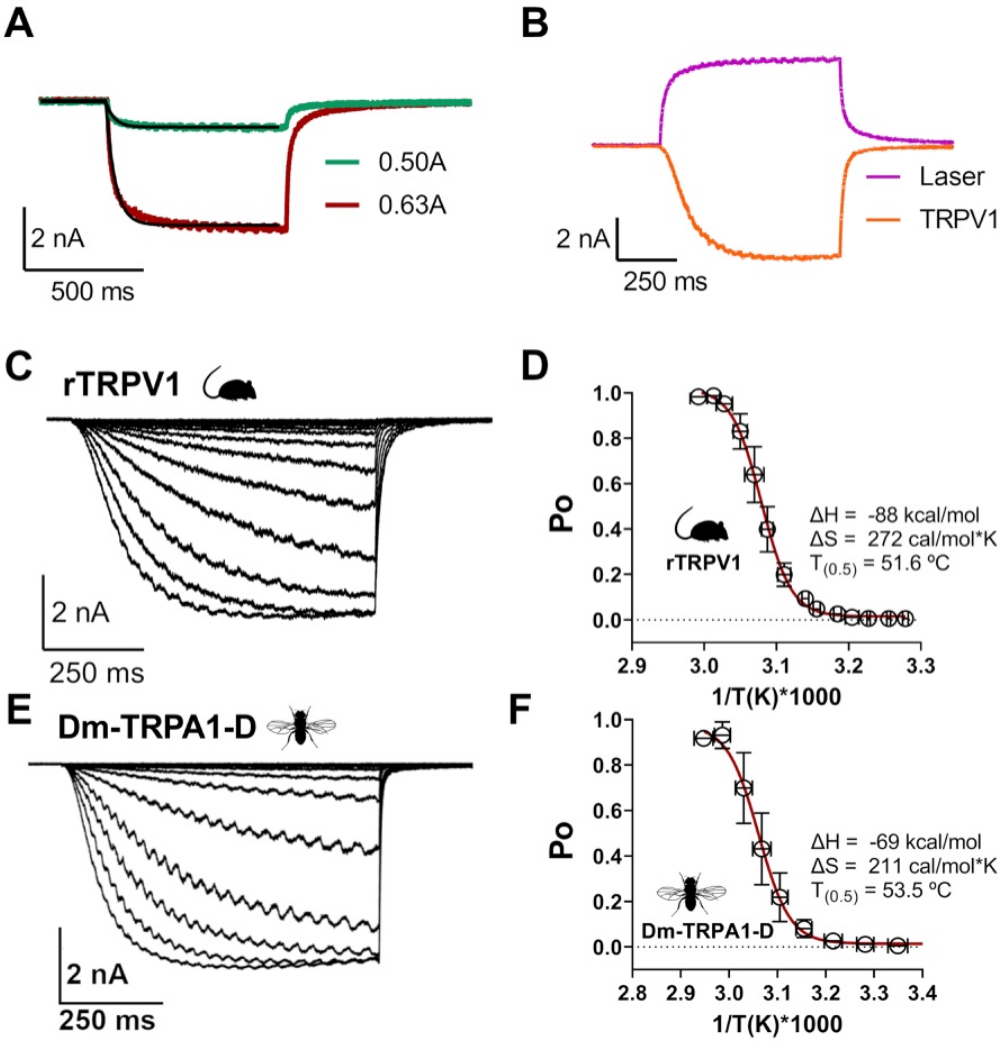
1017 **Figure S6. Schematics of the functional assay workflow and optical fiber-patch clamp recording setup**



1018 **Figure S6. Schematic drawing of the experimental workflow used to characterize *Rhodnius prolixus***
1019 **thermo TRPA5$_2$ ion channel. A**. The full TRPA5$_2$ ORF sequence was amplified from *Rhodnius prolixus* antenna
1020 and gene-specific primers, sequenced-verified, then codon-optimized for mammalian expression, and subcloned
1021 in a custom-made pFRT-TO-FLAG-T2A-mRuby2 expression cassette under expression of the CMV promoter
1022 (1) **(B)**. **C**. HEK293T cells seeded at low density were transiently transfected with plasmid DNA-lipid complexes
1023 and incubated at 37°C for 48h to allow TRP surface membrane expression. Monomeric mRuby2 fluorescent
1024 protein (mRuby2) was co-expressed as a cytoplasmic marker. 48h post-transfection, cells were prepared for
1025 patch-clamp recording by seeding in a 30-mm^2 culture dish overlaid with round glass cover slips. Cells were
1026 incubated at 30°C. **D**. Electrophysiology recordings took place after 24h to 48h using an optical fiber-based setup
1027 adapted after Yao et al 2009 (2), designed to couple manual patch clamp recordings with fiber optics as a mean
1028 to provide controllable optical and thermal stimulations to individual cells expressing candidate thermosensitive
1029 receptor proteins. The setup consists of a fiber launch system combining a high-power optical fiber tuned to
1030 near-infrared wavelengths (λc =1460 nm (+/-20 nm), Po= 4.8 watts), a visible alignment laser (red), and a laser

1031 diode controller, forming a PID control loop using the patch clamp current as the feedback signal. **E**. During the
1032 experiment, a laser spot is aligned with one single patched cell (see Fig. S8) stably expressing the membrane
1033 receptor protein of interest in the cover slip placed in the recording chamber.

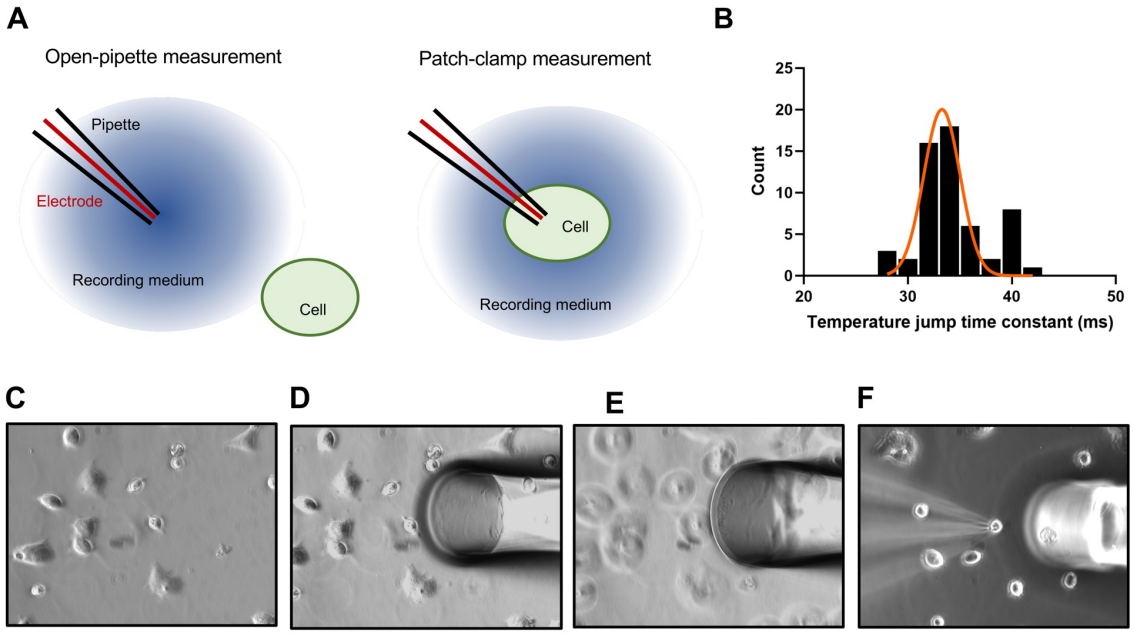
1034 **Figure S7. Validation with rTRPV1 and Dm-TRPA1-D**



1035 **Figure S7. Validation with rTRPV1 and Dm-TRPA1-D**. **A.** Time course of the ionic current through the open
1036 pipette at -10 mV holding voltage when a current of 0.50A (green trace) and 0.63A (red trace) was applied to the
1037 laser diode. The temperature jumps for those currents correspond to 32.1°C and 59.7°C, from 22.6°C,
1038 respectively. Both temperature jumps can be fitted by a mono-exponential function with a time constant of ~35
1039 ms (black line). **B.** Time course comparison between the current through the open pipette (magenta trace) and
1040 a HEK293T cell expressing rTRPV1 channels under voltage clamp at -30 mV (orange trace), in response to a
1041 temperature jump from 22.6°C to 59.7°C (0.63A). The current through rTRPV1 channels is three times slower
1042 than the laser kinetics with a time constant of ~100 ms. **C.** Representative heat-activated current traces of a
1043 HEK293T cell expressing rTRPV1 under voltage clamp (holding voltage of -30 mV). The currents were elicited
1044 by temperature jumps from room temperature (22.6°C) up to 59.7°C with a duration of 700 ms. **D.** Fraction of
1045 rTRPV1 channels in the open state (Open probability, Po) as a function of the temperature. The Po vs 1/T was
1046 fitted to a Boltzman function (red line) with the midpoint of activation ($T_{0.5}$) reached at 51.6°C. The Van't Hoff plot

1047 estimates for rTRPV1 provides an activation enthalpy of the endothermic transition at 88.3±9.4 kcal/mol and an
1048 entropic change associated to the temperature activation process at 271±28 cal/mol*K. at -30 mV as a function
1049 of the temperature. These values in our new expression cassette are very close to previously published values
1050 of ΔH = 85 kcal/mol, $T_{0.5}$ = 45.6°C (2,3). **E.** Representative heat-activated current traces of a HEK293 cell
1051 expressing Dm-TRPA1-D channels under voltage clamp (holding voltage of -30 mV). The currents were elicited
1052 by temperature jumps from room temperature (19.4°C) up to 63.5°C with a duration of 700 ms. **F.** Fraction of
1053 Dm-TRPA1-D channels in the open state (Open probability, Po) as a function of the temperature. The Po vs 1/T
1054 was fitted to a Boltzman function (red line) with the midpoint of activation ($T_{0.5}$) reached at 53.5°C, the Van't Hoff
1055 plot estimates for Dm-TRPA1-D an activation enthalpy of the endothermic transition at 68.7±13.1 kcal/mol and
1056 an entropic change associated to the temperature activation process at 211±40 cal/mol·K at -30 mV. The
1057 stationary current at the end of the temperature pulse (last 100 ms) was used to calculate all the thermodynamics
1058 parameters. Data are presented as means ± standard deviation.

1059 **Figure S8.**



1060

1061 **Figure S8. Open pipette temperature calibration and optic fiber positioning prior to each cell patch clamp**
1062 **experiment A.** Schematic representation of open pipette temperature calibration. First the open pipette is
1063 positioned under the center of the laser using the reference marks on the screen. Then we proceed to record
1064 the current through the open pipette elicited as a function of a series of near IR laser pulses. With these current
1065 traces we can estimate the temperature jumps magnitude that will be applied later to the target cell. After this
1066 the target cell is positioned in the center of the laser beam using the microscope stage translation (the optic fiber
1067 stays fixed). Once the cell is in the right position, we use the same pipette used for the calibration to carry out
1068 the electrophysiological recording, applying the same set of pulses used in the calibration **B.** Histogram of the
1069 time constants from the exponential fit to the open pipette current traces from the temperature jumps used in the
1070 rTRPV1 experiments (n=56), the mean time constant was 33.3±1.8 ms. **C.** View of HEK293T cells seeded at
1071 low density on a glass cover in the recording chamber on the patch-clamp rig station. **D.** After finding a target
1072 cell co-expressing mRuby2 as a fluorescent marker (see Fig. 1B), with the help of an automatized
1073 micromanipulator, the optic fiber is placed in the recording solution using the reference marks on the computer
1074 screen (not shown here) defined during the calibration with the visible laser. The depth in the solution is adjusted
1075 so that the fiber is directly above a single cell in the recording medium. **E.** The field of view is changed to align
1076 the patch clamp electrode to the reference marks. **F.** The target cell expressing mRuby2 is patched with the
1077 recording electrode, now visible on the left side, whereas the fiber is directly above the target cell. The relative
1078 position between the recording pipette and the fiber was established using a visible laser during the setup of the
1079 system, and is constant for all the experiments.

1080 **Extended data References**

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