LETTERS

Analysis of *Drosophila* TRPA1 reveals an ancient origin for human chemical nociception

Kyeongjin Kang[1,2], Stefan R. Pulver[1,2]†, Vincent C. Panzano[1,2], Elaine C. Chang[1,2], Leslie C. Griffith[1,2], Douglas L. Theobald[3] & Paul A. Garrity[1,2]

Chemical nociception, the detection of tissue-damaging chemicals, is important for animal survival and causes human pain and inflammation, but its evolutionary origins are largely unknown. Reactive electrophiles are a class of noxious compounds humans find pungent and irritating, such as allyl isothiocyanate (in wasabi) and acrolein (in cigarette smoke)[1–3]. Diverse animals, from insects to humans, find reactive electrophiles aversive[1–3], but whether this reflects conservation of an ancient sensory modality has been unclear. Here we identify the molecular basis of reactive electrophile detection in flies. We demonstrate that *Drosophila* TRPA1 (Transient receptor potential A1), the *Drosophila melanogaster* orthologue of the human irritant sensor, acts in gustatory chemosensors to inhibit reactive electrophile ingestion. We show that fly and mosquito TRPA1 orthologues are molecular sensors of electrophiles, using a mechanism conserved with vertebrate TRPA1s. Phylogenetic analyses indicate that invertebrate and vertebrate TRPA1s share a common ancestor that possessed critical characteristics required for electrophile detection. These findings support emergence of TRPA1-based electrophile detection in a common bilaterian ancestor, with widespread conservation throughout vertebrate and invertebrate evolution. Such conservation contrasts with the evolutionary divergence of canonical olfactory and gustatory receptors and may relate to electrophile toxicity. We propose that human pain perception relies on an ancient chemical sensor conserved across ~500 million years of animal evolution.

Reactive electrophiles are tissue-damaging agents that modify nucleic acids, proteins and other biomolecules. They are aversive to both vertebrates and invertebrates[1–3]; plants and animals use them as deterrents[3]. Despite their importance as natural repellents, the cellular and molecular mechanisms by which reactive electrophiles deter insects are not well understood. We examined *Drosophila* responses to reactive electrophiles using feeding. When a droplet of food (350 mM sucrose) contacts the legs of a hungry fly, the fly extends its proboscis to drink. This proboscis extension response (PER) is robust and sustained; >90% of the second to fifth offerings of food elicited PER (Fig. 1b). Adding the reactive electrophile allyl isothiocyanate (AITC, Fig. 1a) to the food dramatically inhibited this response (Fig. 1b). This effect was generalized to other reactive electrophiles using *N*-methylmaleimide (NMM) and cinnamaldehyde (CA) (Fig. 1a). Both NMM and CA robustly inhibited feeding (Fig. 1b). This inhibitory effect appeared gustatory, not olfactory, because NMM is non-volatile (melting point 93 °C) and avoidance required ingestion. When only leg contact with food was permitted, reactive electrophiles did not affect PER (Fig. 1c), suggesting that chemosensors along the path of food intake rather than the legs mediate their inhibitory effects. The bitter compound caffeine, for which there are tarsal receptors[4,5], robustly inhibited PER even when ingestion was not permitted (Fig. 1c).

In vertebrates, the cation channel TRPA1 is a molecular receptor for reactive electrophiles, forming covalent adducts with these chemicals and activating sensory neurons to mediate irritation and pain[6–11]. In *Drosophila*, previous *in vitro* physiological analyses of two TRPA1 relatives, *Drosophila* TRPA1 and Painless, suggested these channels were not activated by electrophiles[6,12], raising the possibility that flies might use different mechanisms to detect these

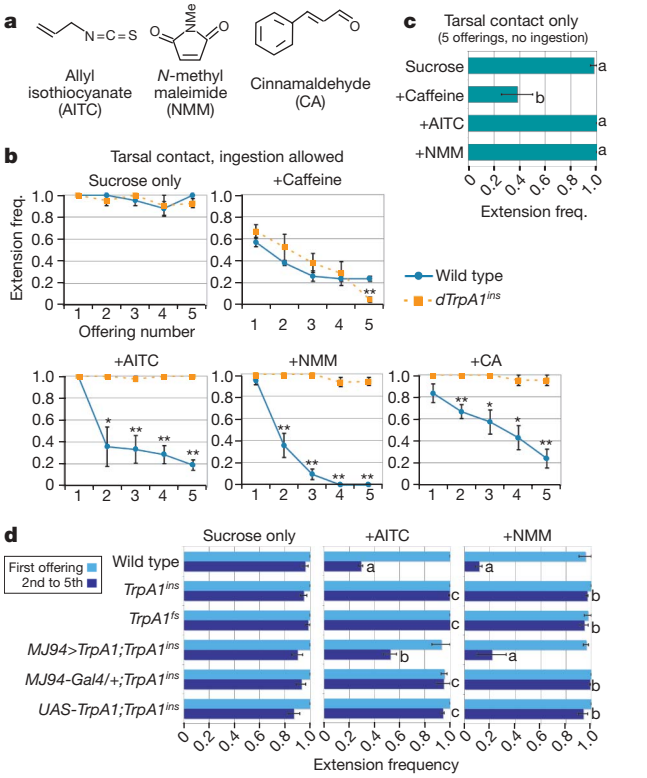

Figure 1 | *Drosophila TrpA1* mediates gustatory responses to reactive electrophiles. a, Chemical structures. **b**, Proboscis extension response (PER) frequency at five sequential tastant offerings, ingestion permitted. (*$P < 0.05$, **$P < 0.01$, unpaired *t*-test.) **c**, PER, tastant contacts only legs. Five sequential offerings combined ($n \geq 10$ flies). **d**, PER, ingestion permitted: light blue, first offering; dark blue, second to fifth offerings combined. Statistically distinct groups marked by different letters (Tukey HSD, $\alpha = 0.01$). Data are mean ±s.e.m. All studies use 12% (350 mM) sucrose, alone or with 100 mM caffeine, 2 mM AITC, 10 mM NMM, or 6 mM CA. $n = 3$ groups of ≥ 7 flies, unless noted.

[1]National Center for Behavioral Genomics, [2]Volen Center for Complex Systems, Department of Biology, [3]Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02454, USA. †Present address: Department of Zoology, University of Cambridge, Cambridge CB2 3EJ, UK.

chemicals. We reexamined the possible involvement of *Drosophila* TRPA1 and Painless *in vivo*, assessing the gustatory response to reactive electrophiles. In contrast to wild type, *Drosophila TrpA1* loss-of-function mutants showed no reduction in PER when offered food containing AITC, NMM or CA (Fig. 1b). Similar defects were observed using two loss-of-function *TrpA1* alleles ($TrpA1^{ins}$ and $TrpA1^{fs}$)[13] and TRPA1 complementary DNA expression rescued this defect (Fig. 1d). Thus this response to reactive electrophiles is entirely TRPA1-dependent. *TrpA1* mutants responded to other deterrents, as caffeine inhibited PER (Fig. 1b). In contrast, *painless* mutants remained responsive to reactive electrophiles (Supplementary Fig. 1), although responses were less robust than controls, suggesting a possible auxiliary function, consistent with previous report[14].

Drosophila TRPA1 protein expression was detected in the mouthparts (Fig. 2a–c), but not legs or labellum. Within the mouthparts, TRPA1 was expressed in neurons innervating sensilla no. 8 and 9 of the labral sense organ (LSO) (Fig. 2b, e). LSO sensilla contain pores that open onto the oesophagus lumen, providing access to chemicals in ingested food. Thus, TRPA1 is expressed in an appropriate place to mediate ingestion-dependent responses.

To test the significance of peripheral *Drosophila* TRPA1 expression, tissue-specific RNA interference (RNAi) was performed using three promoters whose expression overlaps TRPA1-positive LSO neurons: *Dll-Gal4*, expressed broadly within peripheral tissue, *MJ94-Gal4*, expressed in chemoreceptors and the brain[15], and *Gr66a-Gal4*, expressed in chemoreceptors implicated in aversive responses[4,5] (Fig. 2f–i). TRPA1 knockdown using each promoter robustly reduced NMM's effect on PER, consistent with a requirement for TRPA1 in peripheral chemoreceptors (Fig. 2j). In contrast, TRPA1 knockdown in the AC thermosensory neurons of the head using $TrpA1^{SH}$-*Gal4*[16] had no effect (Fig. 2j). These data cleanly distinguish the sites of action for TRPA1 in thermotaxis and gustation, with the former involving AC neurons[16] and the latter peripheral sensory neurons.

Expression of *Drosophila* TRPA1 in peripheral chemosensors also sufficed to induce reactive electrophile-dependent PER inhibition. *TrpA1* cDNA expression with *Dll-Gal4*, *MJ94-Gal4* or *Gr66a-Gal4* rescued the mutant phenotype (Fig. 2k). In addition, ectopic expression of TRPA1 in leg chemoreceptors (using *Gr66a-Gal4*) allowed flies to respond to electrophiles via leg contact (Fig. 2l). Thus, TRPA1 expression in peripheral chemosensory neurons is both necessary and sufficient for reactive-electrophile-induced feeding inhibition.

Drosophila TRPA1 has been considered unresponsive to electrophiles[6,17]; however, we recently found that the original *Drosophila TrpA1* cDNA contained a partially inactivating mutation[16]. Using a wild-type *Drosophila TrpA1* cDNA, we found *Drosophila* TRPA1 was activated by multiple reactive electrophiles when expressed in *Xenopus* oocytes (Fig. 3a–d and Supplementary Figs 2 and 3). TRPA1 orthologues from two other *Drosophila* species, *Drosophila mojavensis* and *Drosophila virilis*, and the malaria mosquito *Anopheles gambiae* also responded to these chemicals (Fig. 3e and Supplementary Fig. 2). Combined with the sensitivity of mosquito TRPA1 to AITC in HEK cells[17], these findings demonstrate multiple insect TRPA1s respond to electrophiles. Notably, electrophile-activated currents persisted after chemical withdrawal (Fig. 3a–f), contrasting with the transient activation of *Drosophila* TRPA1 by warmth[16]. Persistent activation by electrophiles has been observed for mammalian TRPA1s, and it is thought to reflect covalent association between agonists and channel[10,11]. This similarity suggested that reactive electrophiles might activate insect and mammalian TRPA1s via similar mechanisms. Finally, we demonstrated that ectopic expression of *Drosophila* TRPA1 in fly neurons can confer physiological sensitivity to electrophiles. In contrast to controls or motor neurons expressing Painless, TRPA1-expressing motor neurons were CA-responsive (Fig. 3f and Supplementary Fig. 4). Thus, TRPA1 acts as an electrophile sensor in multiple contexts.

Reactive electrophiles activate mammalian TRPA1s by forming covalent bonds with cysteine and lysine residues in the channel; six residues (five cysteines and one lysine) are implicated in electrophile detection, and mutations in these residues decrease electrophile sensitivity[10,11]. Insect TRPA1s conserve five of these six residues (Fig. 4a). Mutating *Drosophila* TRPA1 cysteines 650 and 670 to serines (TRPA1-2C) significantly decreased AITC sensitivity (Fig. 4b, c); this TRPA1-2C mutant remained robustly warmth-activated (Supplementary Fig. 5). The shared requirement for these residues further supports a common mechanism for reactive electrophile sensing by fly and vertebrate TRPA1s. TRPA1s also exhibit some species-specific differences in chemical sensitivity; 2-aminoethoxydiphenyl borate (2-APB) and nicotine, conserved cysteine-independent agonists of mammalian TRPA1s[10,18], did not activate *Drosophila* TRPA1 (Supplementary Fig. 6).

While functional similarities between insect and vertebrate TRPA1s could reflect conservation of an ancestral mechanism for electrophile detection, the electrophile insensitivity of invertebrate

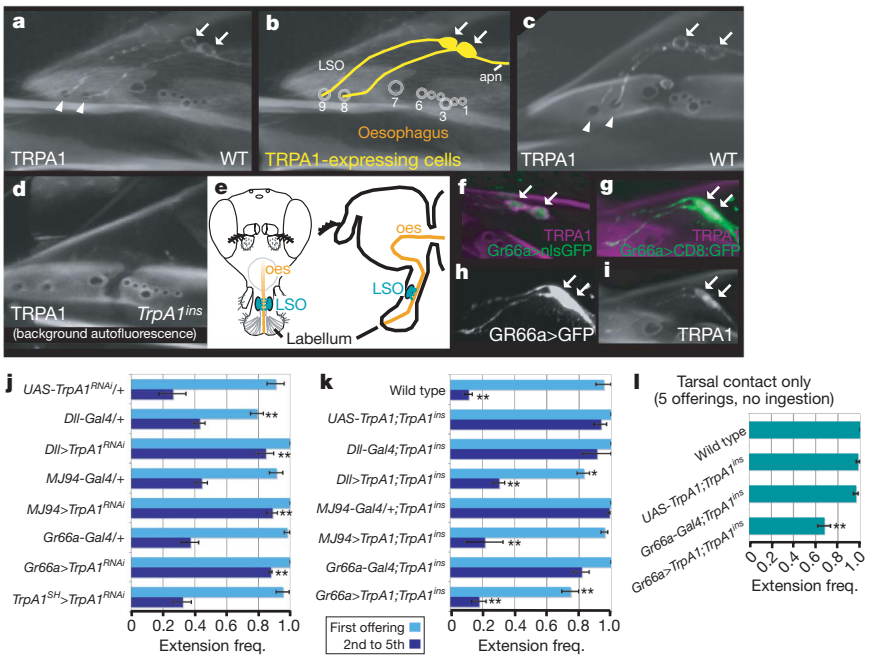

Figure 2 | *Drosophila TrpA1* functions in chemosensors. **a**–**c**, TRPA1 expression. Arrows denote cell bodies, arrowheads distal neurites. Cuticle autofluorescence visible. In **b**, LSO sensilla are numbered. apn, accessory pharyngeal nerve. **d**, *TrpA1* mutants lack TRPA1 staining. **e**, oes, oesophagus. **f**–**i**, *Gr66a-Gal4* and TRPA1 co-expression in LSO. **f**–**h**, Nuclear (**f**) and membrane (**g**, **h**) green fluorescent protein (GFP) expressed using *Gr66a-Gal4*. **j**–**l**, PER to 350 mM sucrose containing 10 mM NMM; ingestion permitted (**j**, **k**), tarsal contact only (**l**). **j**, TRPA1 knockdown. **k**, TRPA1 rescue. **l**, TRPA1 gain-of-function. *$\alpha = 0.05$, **$\alpha = 0.01$, differ from Gal4 and UAS controls, Tukey HSD. **j**, **k**, $n = 3$ groups of 7–8 flies; **l**, $n \geq 10$ flies. Field of view widths: **a**, **b**, 120 μm; **c**, 80 μm; **d**, 85 μm; **f**, 40 μm. **g**–**i**, 50 μm.

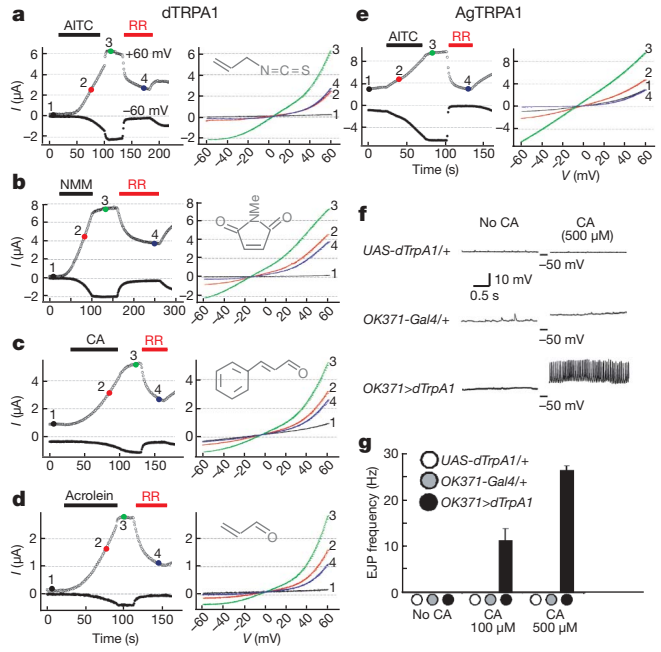

Figure 3 | Insect TRPA1s are reactive electrophile sensors. **a**–**e**, Representative responses of *Drosophila* TRPA1 (**a**–**d**) and *Anopheles gambiae* TRPA1 (**e**) expressed in oocytes. Left panels, currents at −60 and +60 mV. Perfusion buffer containing indicated chemical (**a**, **c**, **d**, 100 μM; **b**, **e**, 40 μM) was applied for 60–80 s. 100 μM ruthenium red (RR) applied as noted. Right panels show current–voltage (*I*–*V*) relationships at points marked on left panels. **f**, **g**, Ectopic *Drosophila* TRPA1 expression confers electrophile sensitivity upon motor neurons. **f**, Motor-neuron-driven excitatory junction potentials (EJPs) from third instar larval muscles. **g**, Mean EJP frequencies. In controls, no EJPs were observed.

TRPA1 relatives like Painless[12] and *Caenorhabditis elegans* TRPA1 (ref. 19) raised the possibility that some insect and vertebrate TRPA1s recently converged on similar mechanisms. To test these alternatives, a phylogeny of TRPA proteins was constructed using three different approaches—Bayesian inference[20], maximum likelihood[21] and neighbour joining[22]. Trees were rooted using TRPAs from the unicellular choanoflagellate *Monosiga brevicollis*. All methods indicated with high confidence that the electrophile-activated TRPA1 channels of invertebrates and vertebrates belong to a monophyletic clade, the TRPA1 clade, distinct from other TRPAs (termed basal TRPAs) by both tree topology and branch lengths (Fig. 4d, e and Supplementary Figs 7–9). The TRPA1 clade channels derive from a common ancestral TRPA1 present in the common ancestor of vertebrates and invertebrates (Fig. 4d, e). Consistent with a common evolutionary origin of electrophile detection, sequence reconstruction[23] suggested this ancestral TRPA1 contained all six critical residues associated with electrophile sensing (Fig. 4f). This mode of electrophile detection appears specific to TRPA1 clade members, as no known basal TRPAs conserve more than one of the five cysteines implicated in electrophile detection (Fig. 4a).

These analyses also suggest revisions to proposed relationships among TRPAs. Painless has been called the fly homologue of mammalian TRPA1, and *C. elegans* TRPA1 considered the nematode TRPA1 orthologue. However, all analyses indicated that neither Painless nor *C. elegans* TRPA1 descend from the ancestral TRPA1; both are closer to anemone and choanoflagellate TRPAs (Fig. 4d). Consistent with their electrophile insensitivity[12,19], Painless and *C. elegans* TRPA1 lack most cysteines implicated in electrophile detection (Fig. 4a). During evolution, nematodes appear to have lost their TRPA1 orthologue and vertebrates their basal TRPA(s) (Fig. 4d, e).

Functional conservation of TRPA1 provides a simple molecular foundation for the widespread aversion to reactive electrophiles across the animal kingdom. The conservation of reactive electrophile detection differs from other chemical senses like olfaction and gustation whose origins are molecularly diverse and evolutionarily distinct[24,25]. For example, many fly olfactory receptors are ion channels rather than the G-protein-coupled receptors of vertebrates[25]. Reactive electrophile detection also contrasts with capsaicin detection; capsaicin activates mammalian nociceptors[1], but elicits no

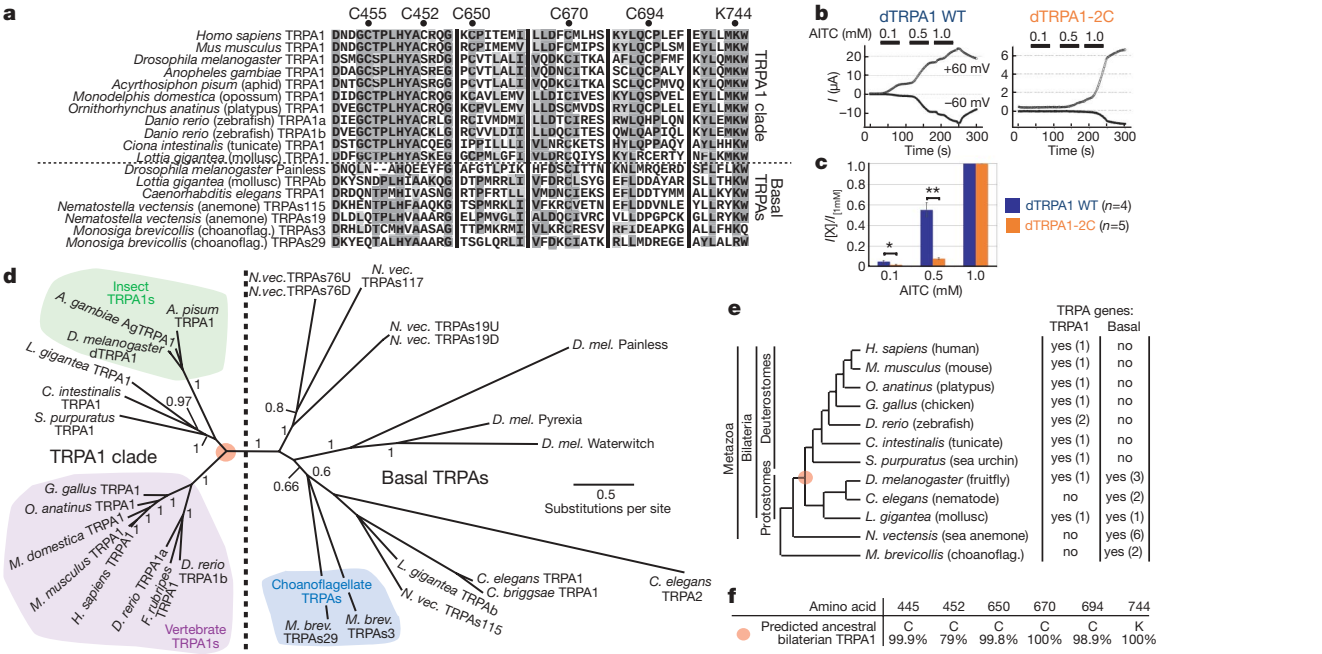

Figure 4 | TRPA phylogeny. **a**, Conservation of residues implicated in electrophile detection. Choannoflag., choanoflagellate. **b**, *Drosophila* TRPA1-WT (wild type) and *Drosophila* TRPA1-2C channels in *Xenopus* oocytes. 60 s pulses of AITC (0.1, 0.5 and 1.0 mM) were applied with 25 s intervals. **c**, +60 mV currents normalized to channel's response to 1.0 mM AITC. $*P < 0.05$, $**P < 0.001$, unpaired *t*-test. **d**, Bayesian consensus phylogeny for TRPAs. Internal branches labelled by posterior probability (<0.5 branches collapsed). Red dot denotes ancestor of TRPA1 clade. **e**, Cladogram showing TRPA complements, including numbers of channels. Red dot denotes bilaterian ancestor. **f**, PAML residue identity estimates for ancestral TRPA1.

acute response in flies or nematodes. The exceptional conservation of TRPA1-mediated nociception could relate to the toxicity of reactive electrophiles[26], which could provide selective pressure for maintaining an effective monitoring system.

The ability of *Drosophila* TRPA1 to mediate aversive responses to natural deterrents suggests insect TRPA1s as targets for developing new deterrents. Insect TRPA1 agonists could be useful against an array of pests, as disease vectors from mosquitoes to lice and agricultural pests from flour beetles to aphids contain *Drosophila* TRPA1 relatives[16]. While natural electrophilic deterrents also activate mammalian TRPA1s, deterring insects and mammals alike, the differential responsiveness of insect and mammalian TRPA1 s to other stimuli like temperature[27] and nicotine raises the possibility of identifying insect-specific TRPA1 agonists. Such selective agonists could maximize pest deterrence while minimizing irritation to other animals.

TRPA1 activation by reactive electrophiles is a key component of mammalian nociceptor function, eliciting pungency, irritation, inflammation and pain[6–9]. Our findings demonstrate that the molecular mechanism that initiates these perceptions is not a recent evolutionary innovation and that it is not specific to vertebrates. Rather, we propose that reactive electrophile detection represents an ancient sensory modality conserved in molecular detail across ~500 million years of animal evolution.

METHODS SUMMARY

Proboscis extension behaviour. The proboscis extension assay was modified from ones previously described[4,5] as detailed in Methods.

Physiology. Oocyte and larval physiology were performed largely as described[16,28], with additional details provided in Methods. Chemical sensitivities of wild type and mutant (*Drosophila* TRPA1-2C) channels were assessed by normalizing all currents to currents observed at 1 mM AITC. Chemically unrelated insect repellents like DEET, IR-3535 and deltamethrin failed to activate TRPA1 (K.K. and P.A.G., unpublished data).

Phylogeny. TRPA sequences were assembled from available genomic and EST data. Multiple sequence alignment was performed using ProbCons[29] for the region from ~310 amino acids amino-terminal of transmembrane regions (containing the residues implicated in chemical sensing) to ~50 amino acids carboxy-terminal of transmembrane regions (Supplementary Fig. 9). Bayesian analysis was calculated with the parallel version of MrBayes 3.1.2 using mixed substitution rate matrices and gamma distributed rate variation across sites (8 categories). An exponential prior (mean = 1.0) was assumed for the shape parameter of the gamma distribution, an unconstrained exponential prior (mean = 1.0) assumed for branch lengths, and a uniform prior assumed for all labelled topologies. Two independent MCMC analyses were performed (each with one cold and three heated chains), with other parameters set to defaults. Chains were run for 10,000,000 generations, and convergence inferred after cold chain topologies reached a standard deviation of split frequencies of less than 0.005 (~250,000 generations). After convergence, the first half of the chain was discarded as 'burn-in'. Maximum likelihood analysis was performed with PhyML 3.0, using an LG substitution rate matrix, and gamma distributed rate variation (8 categories) and was bootstrapped 1,000 times. A BioNJ distance-based phylogenetic analysis was performed with PAUP 4b10 (ref. 30) and bootstrapped 1,000 times. Ancestral sequence reconstruction was performed with PAML 4.2b[23] using the consensus Bayesian phylogenetic tree and mean alpha rate parameter. Branch lengths were fixed.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 20 November 2009; accepted 21 January 2010.
Published online 17 March 2010.

1. Basbaum, A. I., Bautista, D. M., Scherrer, G. & Julius, D. Cellular and molecular mechanisms of pain. *Cell* **139,** 267–284 (2009).
2. Bessac, B. F., Jordt, S. E. & Breathtaking, T. R. P. Channels: TRPA1 and TRPV1 in airway chemosensation and reflex control. *Physiology* **23,** 360–370 (2008).
3. Eisner, T. in *Chemical Ecology* (eds Sondheimer, E. & Simeone, J. B.) 157–218 (Academic, 1970).
4. Thorne, N., Chromey, C., Bray, S. & Amrein, H. Taste perception and coding in *Drosophila. Curr. Biol.* **14,** 1065–1079 (2004).
5. Wang, Z., Singhvi, A., Kong, P. & Scott, K. Taste representations in the *Drosophila* brain. *Cell* **117,** 981–991 (2004).
6. Bandell, M. *et al.* Noxious cold ion channel TRPA1 is activated by pungent compounds and bradykinin. *Neuron* **41,** 849–857 (2004).
7. Jordt, S. E. *et al.* Mustard oils and cannabinoids excite sensory nerve fibres through the TRP channel ANKTM1. *Nature* **427,** 260–265 (2004).
8. Bautista, D. M. *et al.* TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents. *Cell* **124,** 1269–1282 (2006).
9. Kwan, K. Y. *et al.* TRPA1 contributes to cold, mechanical, and chemical nociception but is not essential for hair-cell transduction. *Neuron* **50,** 277–289 (2006).
10. Hinman, A., Chuang, H. H., Bautista, D. M. & Julius, D. TRP channel activation by reversible covalent modification. *Proc. Natl Acad. Sci. USA* **103,** 19564–19568 (2006).
11. Macpherson, L. J. *et al.* Noxious compounds activate TRPA1 ion channels through covalent modification of cysteines. *Nature* **445,** 541–545 (2007).
12. Sokabe, T., Tsujiuchi, S., Kadowaki, T. & Tominaga, M. *Drosophila* painless is a Ca^{2+}-requiring channel activated by noxious heat. *J. Neurosci.* **28,** 9929–9938 (2008).
13. Rosenzweig, M., Kang, K. & Garrity, P. A. Distinct TRP channels are required for warm and cool avoidance in *Drosophila melanogaster. Proc. Natl Acad. Sci. USA* **105,** 14668–14673 (2008).
14. Al-Anzi, B., Tracey, W. D. Jr & Benzer, S. Response of *Drosophila* to wasabi is mediated by painless, the fly homolog of mammalian TRPA1/ANKTM1. *Curr. Biol.* **16,** 1034–1040 (2006).
15. Gendre, N. *et al.* Integration of complex larval chemosensory organs into the adult nervous system of *Drosophila. Development* **131,** 83–92 (2004).
16. Hamada, F. N. *et al.* An internal thermal sensor controlling temperature preference in *Drosophila. Nature* **454,** 217–220 (2008).
17. Xiao, B. *et al.* Identification of transmembrane domain 5 as a critical molecular determinant of menthol sensitivity in mammalian TRPA1 channels. *J. Neurosci.* **28,** 9640–9651 (2008).
18. Talavera, K. *et al.* Nicotine activates the chemosensory cation channel TRPA1. *Nature Neurosci.* **12,** 1293–1299 (2009).
19. Kindt, K. S. *et al. Caenorhabditis elegans* TRPA-1 functions in mechanosensation. *Nature Neurosci.* **10,** 568–577 (2007).
20. Ronquist, F. & Huelsenbeck, J. P. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19,** 1572–1574 (2003).
21. Guindon, S., Delsuc, F., Dufayard, J. F. & Gascuel, O. Estimating maximum likelihood phylogenies with PhyML. *Methods Mol. Biol.* **537,** 113–137 (2009).
22. Saitou, N. & Nei, M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4,** 406–425 (1987).
23. Yang, Z. PAML 4: phylogenetic analysis by maximum likelihood. *Mol. Biol. Evol.* **24,** 1586–1591 (2007).
24. Bargmann, C. I. Comparative chemosensation from receptors to ecology. *Nature* **444,** 295–301 (2006).
25. Nakagawa, T. & Vosshall, L. B. Controversy and consensus: noncanonical signaling mechanisms in the insect olfactory system. *Curr. Opin. Neurobiol.* **19,** 284–292 (2009).
26. Gomes, R., Meek, M. E. & Eggleton, M. *Concise International Chemical Assessment Document No 43* (World Health Organization, Geneva, 2002).
27. Viswanath, V. *et al.* Opposite thermosensor in fruitfly and mouse. *Nature* **423,** 822–823 (2003).
28. Pulver, S. R., Pashkovski, S. L., Hornstein, N. J., Garrity, P. A. & Griffith, L. C. Temporal dynamics of neuronal activation by Channelrhodopsin-2 and TRPA1 determine behavioral output in *Drosophila* larvae. *J. Neurophysiol.* **101,** 3075–3088 (2009).
29. Do, C. B., Mahabhashyam, M. S., Brudno, M. & Batzoglou, S. ProbCons: probabilistic consistency-based multiple sequence alignment. *Genome Res.* **15,** 330–340 (2005).
30. Swofford, D. L. *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods)* version 4 (Sinauer Associates, Sunderland, MA, 2003).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank H. Amrein, G. Boekh, K. Scott, D. Tracey and Bloomington Stock center for flies, C. Miller-Patterson and D. Zeiger for assistance, and members of the Garrity laboratory, J. P. Garrity, L. Huang, E. Marder, S. Nelson, G. Turrigiano and M. Rosbash for comments. This work was supported by grants from the NIMH (R21 MH080206, P.A.G.; RO1 MH067284, L.C.G.), NINDS (PO1 NS044232), and the Royal Society (Newton International Fellowship, S.R.P.).

Author Contributions K.K., S.R.P., V.C.P., D.L.T. and P.A.G. designed experiments. K.K. performed molecular genetics, behaviour and oocyte physiology. S.R.P. performed neuromuscular junction electrophysiology. E.C.C. assisted with behaviour, D.L.T., V.C.P. and P.A.G. performed bioinformatics, and K.K. and P.A.G. wrote the paper with assistance from S.R.P., V.C.P., L.C.G. and D.L.T.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to P.A.G. (pgarrity@brandeis.edu).

METHODS

Fly strains and immunohistochemistry. *Drosophila TrpA1^{SH}-Gal4, UAS-TRPA1* and *UAS-TRPA1^{dsRNA}* transgenic strains[16], as well as *Dll-Gal4* (ref. 31), *MJ94-Gal4* (refs 15, 32), *Gr66a-Gal4* (ref. 33), *UAS-Painless^{AR9}* (ref. 14) and *painless*[34] mutants have been previously described. *UAS-nls:GFP* and *UAS-mCD8:GFP* fly strains were obtained from Bloomington. Anti-*Drosophila* TRPA1 immunohistochemistry was performed as described[35]. Details of the creation of *TrpA1^{fs}* and *TrpA1^{ins}* were previously reported[13,16]. Briefly, *TrpA1^{fs}* has a 2-base-pair (bp) insertion creating frameshift mutation within the third ankyrin repeat of TRPA1, before the transmembrane regions. *TrpA1^{ins}* contains two mutated copies of *TrpA1* that flank vector targeting sequences: one copy lacks the ion pore and sixth transmembrane domain, while the other copy lacks the promoter and upstream sequences, all of exon 1, part of exon 2, and contains the 2-bp insertion mutation present in *TrpA1^{fs}*.

PER behavioural assays. Two to seven day old flies were starved overnight on wet Kim wipes, anaesthetized on ice, and affixed to a glass slide. Flies recovered in a humidified chamber for at least 2 h at room temperature before testing. During the PER assay, the fly was first satiated with water, then a solution containing tastants was touched to the forelegs as a liquid ball on a pipette tip. If the proboscis was extended and contact with the food was maintained for 2–3 s, the response was scored as 1. If the proboscis stuttered on the tastant, or contact was brief, a 0.5 was awarded. If the proboscis failed to contact the solution within 5 s of offering, a 0 was awarded. Each fly was offered tastants five times per experiment, and between offerings water was given to satiation. Because AITC, cinnamaldehyde and NMM were usually accepted on first offering, PER frequency was calculated for the second through to the fifth offerings (sum of four scores per fly divided by 4). Responses to sucrose resumed within ~10 min after pungent chemical exposure, indicating that feeding was not permanently impaired (K.K. and P.A.G., unpublished results). For leg only PER assays, the procedures were as above except flies were not allowed to contact the food with their proboscis. We found that NMM had no effect on ingestion when using a previously published ingestion-independent PER assay for chemical sensitivity[14], suggesting the inhibitory effects of AITC in that assay were not gustatory.

Physiology. Two-electrode voltage clamping on *Xenopus laevis* oocytes. Agonist-evoked *Drosophila* TRPA1 currents were recorded as previously described[16], with the following modifications. Agonists of interest were added to the oocyte perfusion buffer (96 mM NaCl, 1 mM $MgCl_2$, 4 mM KCl and 5 mM HEPES, pH 7.6). Voltage was initially held at −60 mV, and a 300-ms voltage ramp (−60 mV to 60 mV) per second was applied to *Drosophila* TRPA1- or *A. gambiae* TRPA1-expressing oocytes during perfusion of agonist-containing buffer. Typical oocyte resting membrane potentials were between −25 and −60 mV. Agonist-elicited currents were specific and TRPA1-dependent; they were absent from uninjected or water-injected oocytes and were significantly reduced by mutation of two cysteine residues within *Drosophila* TRPA1 (Figs 3a–e, 4b and data not shown). Furthermore, they were inhibited by ruthenium red, which partially inhibits warmth-activated *Drosophila* TRPA1 and *A. gambiae* TRPA1 currents, and they exhibited the reversal potential and rectification properties previously associated with warmth-activated *Drosophila* TRPA1 and *A. gambiae* TRPA1 currents[16]. EC_{50} values for wild-type *Drosophila* TRPA1 channels were obtained at −60 mV, with AITC provided for 60 s with 30 s intervals between increasing concentrations. The low sensitivity of *Drosophila* TRPA1-2C to AITC precluded EC_{50} analysis of the mutant channel.

Larval neuromuscular junction electrophysiology. TRPA channels were expressed in larval motor neurons using OK371-GAL4, a driver specific for glutamatergic neurons, as described[28]. In all preparations, the ventral ganglion was dissected away, leaving only motor axons and terminals. Larval muscle 6 (m6) was impaled with a sharp electrode (10–20 MΩ) containing 3 M KCl. Resting membrane potentials were typically between −40 and −50 mV. Saline was perfused over the preparation, then increasing concentrations of cinnamaldehyde applied using a custom built gravity perfusion system. EJP frequency was measured ~30 s after application of each concentration using analysis scripts in Spike 2 (Cambridge Electronic Design). Painless was overexpressed using the functional rescue construct *UAS-Painless^{AR9}* (ref. 14).

Molecular biology. Substitutions of cysteine/lysine residues in *Drosophila* TRPA1 were made by swapping a region of wild-type cDNA sequence including codons of cysteine or lysine with mutated cassettes. A pair of mutually complementary oligonucleotide primers with a desired mutation were prepared, and each of them was paired with upstream or downstream primers for the first two PCR reactions. The resulting two PCR fragments overlap in the mutant primer-annealing region that contains the replaced codons, and served as template for the second PCR reaction amplified only with the upstream and downstream primers. The upstream and downstream primers were designed to be just outside of specific restriction endonuclease target sites that were used to clone the second PCR products back in the wild-type *Drosophila TrpA1* cDNA background sequence. The fragments amplified by PCR were confirmed by sequencing after cloning to make sure that only desired mutations were introduced in the final cDNA constructs.

Sequence alignment and phylogeny. Multiple sequence alignments were visualized using JAL2.4 (ref. 36). Conservation reflects conservation of physico-chemical properties of residues calculated as described[37]. Quality is inversely proportional to the cost of mutations in a residue, measure of likelihood of observing mutations[36]. Consensus reflects percentage of modal residue.

The LG substitution matrix was as described[38]. The input data for the ancestral reconstruction was the consensus Bayesian phylogenetic tree depicted in Fig. 4d. We used the 'marginal reconstruction' method (RateAncestor = 1) in PAML4 (ref. 25), which determines the posterior probability of each amino acid at each site in the protein alignment for a given node. We fixed the alpha parameter (for gamma distributed rate variation across sites) to the Bayesian expected value as determined by MrBayes.

31. Calleja, M., Moreno, E., Pelaz, S. & Morata, G. Visualization of gene expression in living adult Drosophila. *Science* **274,** 252–255 (1996).
32. Joiner Ml, A. & Griffith, L. C. CaM kinase II and visual input modulate memory formation in the neuronal circuit controlling courtship conditioning. *J. Neurosci.* **17,** 9384–9391 (1997).
33. Dunipace, L., Meister, S., McNealy, C. & Amrein, H. Spatially restricted expression of candidate taste receptors in the Drosophila gustatory system. *Curr. Biol.* **11,** 822–835 (2001).
34. Tracey, W. D. Jr, Wilson, R. I., Laurent, G. & Benzer, S. *painless*, a *Drosophila* gene essential for nociception. *Cell* **113,** 261–273 (2003).
35. Rosenzweig, M. *et al.* The *Drosophila* ortholog of vertebrate TRPA1 regulates thermotaxis. *Genes Dev.* **19,** 419–424 (2005).
36. Waterhouse, A. M., Procter, J. B., Martin, D. M. A., Clamp, M. & Barton, G. J. Jalview version 2: a multiple sequence alignment and analysis workbench. *Bioinformatics* **25,** 1189–1191 (2009).
37. Livingstone, C. D. & Barton, G. J. Protein sequence alignments: a strategy for the hierarchical analysis of residue conservation. *Comput. Applic. Biosci.* **9,** 745–756 (1993).
38. Le, S. Q. & Gascuel, O. An improved general amino acid replacement matrix. *Mol. Biol. Evol.* **25,** 1307–1320 (2008).