Immunoprecipitation

See Supplementary Information for the detailed protocol. In brief, cells were lysed in Nonidet P-40 (NP-40) buffer and cleared by centrifugation at 4 °C. Antibody-conjugated agarose was added to the supernatant and incubated at 4 °C for 3 h or overnight. Beads were washed four times in NP-40 buffer plus 500 mM NaCl. Co-immunprecipitations were treated in the same way, except that the beads were washed in NP-40 buffer without salt.

Immunofluorescence

Cells were washed with PBS and fixed in 3.7% formaldehyde. Permeabilization, washes and antibody dilutions were done with 1% calf serum and 0.25% Triton X-100 in PBS. Primary antibodies were diluted 1:80. FITC-conjugated goat anti-mouse was used at a dilution of 1:300, and goat anti-rabbit was used at 1:1,000. For the experiments in Fig. 4f, the cells were washed with PBS then incubated in cold 0.1% Triton in PBS for 10 min at 4 °C. The cells were then fixed and immunostained as described above. All images were captured at a magnification of ×40 on a confocal microscope (Leica).

For fluorescence *in situ* ERK activation assays (Fig. 4e), cells were stained with antibodies against phosphorylated ERK1/2 (Cell Signalling, diluted 1:100) and antibodies against ERK1 (Santa Cruz, 1:100), followed by AlexaFluor-680-conjugated anti-mouse (Molecular Probes, 1:100) and IRDye800-conjugated anti-rabbit (Rockland, 1:100) secondary antibodies. Signal detection and quantification were done by the Odyssey system (Li-Cor) as directed by the manufacturer.

In situ ERK activation assay

We infected KSR1$^{-/-}$ cells with bicistronic retroviruses encoding KSR1–IRES–GFP and IMP–IRES–YPF or control viruses, and sorted them for green and yellow fluorescence by FACS. Sorted cells were seeded at 1.5×10^4 cells per well in 96-well plates 24 h before analysis. Cells at 70% confluence were deprived of serum for 4 h, and treated with 100 ng ml^{-1} EGF or 25 ng ml^{-1} PDGF for 5 min, before being assayed for ERK activation by a quantitative fluorescence *in situ* plate assay.

Phosphatase and Raf kinase assays

See Supplementary Information for the phosphatase assays. For the Raf kinase assays, Raf1 or RafBXB was immunoprecipitated and washed five times in NP-40 buffer, once in PBS, and once in 25 mM HEPES (pH 7.5) and 10 mM $MgCl_2$. The beads were then added to kinase assay reagents comprising 10 mM $MgCl_2$, 83 μM ATP, 25 mM HEPES (pH 7.5) and 50 ng μl^{-1} recombinant MEK(K94A). The reaction was incubated at 30 °C for 40 min with regular mixing, and stopped on the addition of 2 × sample buffer.

Received 1 October; accepted 14 November 2003; doi:10.1038/nature02237.

1. Nguyen, A. *et al.* Kinase suppressor of Ras (KSR) is a scaffold which facilitates mitogen-activated protein kinase activation *in vivo*. *Mol. Cell. Biol.* **22,** 3035–3045 (2002).
2. Jensen, K., Shiels, C. & Freemont, P. S. PML protein isoforms and the RBCC/TRIM motif. *Oncogene* **20,** 7223–7233 (2001).
3. Li, S. *et al.* Identification of a novel cytoplasmic protein that specifically binds to nuclear localization signal motifs. *J. Biol. Chem.* **273,** 6183–6189 (1998).
4. White, M. A. *et al.* Multiple Ras functions can contribute to mammalian cell transformation. *Cell* **80,** 533–541 (1995).
5. Bruder, J. T., Heidecker, G. & Rapp, U. R. Serum-, TPA-, and Ras-induced expression from Ap-1/Ets-driven promoters requires Raf-1 kinase. *Genes Dev.* **6,** 545–556 (1992).
6. Diaz, B. *et al.* Phosphorylation of Raf-1 serine 338/serine 339 is an essential regulatory event for Ras-dependent activation and biological signalling. *Mol. Cell. Biol.* **17,** 4509–4516 (1997).
7. Joazeiro, C. A. & Weissman, A. M. RING finger proteins: mediators of ubiquitin ligase activity. *Cell* **102,** 549–552 (2000).
8. Yeung, K. *et al.* Suppression of Raf-1 kinase activity and MAP kinase signalling by RKIP. *Nature* **401,** 173–177 (1999).
9. Muller, J. S. O., Copeland, T., Piwnica-Worms, H. & Morrison, D. K. C-TAK1 regulates Ras signalling by phosphorylating the MAPK scaffold, KSR1. *Mol. Cell* **8,** 983–993 (2001).
10. Roy, F., Laberge, G., Douziech, M., Ferland-McCollough, D. & Therrien, M. KSR is a scaffold required for activation of the ERK/MAPK module. *Genes Dev.* **16,** 427–438 (2002).
11. Brennan, J. A., Volle, D. J., Chaika, O. V. & Lewis, R. E. Phosphorylation regulates the nucleocytoplasmic distribution of kinase suppressor of Ras. *J. Biol. Chem.* **277,** 5369–5377 (2002).
12. Sundaram, M. & Han, M. The *C. elegans* KSR-1 gene encodes a novel Raf-related kinase involved in Ras-mediated signal transduction. *Cell* **83,** 889–901 (1995).
13. Stewart, S. *et al.* Kinase suppressor of Ras forms a multiprotein signalling complex and modulates MEK localization. *Mol. Cell. Biol.* **19,** 5523–5534 (1999).
14. Clemens, J. C. *et al.* Use of double-stranded RNA interference in *Drosophila* cell lines to dissect signal transduction pathways. *Proc. Natl Acad. Sci. USA* **97,** 6499–6503 (2000).
15. Elbashir, S. M. *et al.* Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411,** 494–498 (2001).
16. Chien, Y. & White, M. A. RAL GTPases are linchpin modulators of human tumour-cell proliferation and survival. *EMBO Rep.* **4,** 800–806 (2003).

Supplementary Information accompanies the paper on **www.nature.com/nature**.

Acknowledgements This work was supported by grants from the National Cancer Institute (to M.A.W. and to R.E.L.) and the Welch Foundation, and by an Idea Development award (to M.A.W.).

Competing interests statement The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to M.A.W. (michael.white@utsouthwestern.edu).

Mustard oils and cannabinoids excite sensory nerve fibres through the TRP channel ANKTM1

Sven-Eric Jordt[1], Diana M. Bautista[1], Huai-hu Chuang[1], David D. McKemy[1], Peter M. Zygmunt[3], Edward D. Högestätt[3], Ian D. Meng[2]* & David Julius[1]

[1]*Department of Cellular and Molecular Pharmacology and* [2]*Department of Neurology, University of California, San Francisco, California 94143-2140, USA*
[3]*Department of Clinical Pharmacology, Institute of Laboratory Medicine, Lund University Hospital, SE-221 85 Lund, Sweden*

* Present address: Department of Physiology, College of Osteopathic Medicine, University of New England, 11 Hills Beach Road, Biddeford, Maine 04005, USA

Wasabi, horseradish and mustard owe their pungency to isothiocyanate compounds. Topical application of mustard oil (allyl isothiocyanate) to the skin activates underlying sensory nerve endings, thereby producing pain, inflammation and robust hypersensitivity to thermal and mechanical stimuli[1,2]. Despite their widespread use in both the kitchen and the laboratory, the molecular mechanism through which isothiocyanates mediate their effects remains unknown. Here we show that mustard oil depolarizes a subpopulation of primary sensory neurons that are also activated by capsaicin, the pungent ingredient in chilli peppers, and by Δ^9-tetrahydrocannabinol (THC), the psychoactive component of marijuana. Both allyl isothiocyanate and THC mediate their excitatory effects by activating ANKTM1, a member of the transient receptor potential (TRP) ion channel family recently implicated in the detection of noxious cold[3,4]. These findings identify a cellular and molecular target for the pungent action of mustard oils and support an emerging role for TRP channels as ionotropic cannabinoid receptors[5–8].

Capsaicin and mustard oil are plant-derived natural products that elicit pain and inflammation when applied to the skin[1,2]. These effects are produced by the depolarization of sensory nerve fibres that detect noxious stimuli (so-called nociceptors). In addition to transmitting 'pain signals' to the spinal cord, nociceptors may also release peptides—such as substance P and calcitonin-gene-related peptide (CGRP)—peripherally to produce vascular leakage and vasodilation, leading to inflammation and tenderness at the site of irritant application[9,10]. Capsaicin mediates these effects by activating the vanilloid receptor TRPV1, a heat-sensitive cation channel on nociceptor terminals[11–13]. TRPV1 belongs to the greater family of TRP channels, many of which are key components of signal transduction pathways in a variety of sensory systems[14–16].

Topical administration of mustard oil also produces nociceptor excitation, but whether this occurs through a direct action on sensory neurons or through a specific membrane receptor is not known[17]. TRPV1-deficient mice retain sensitivity to mustard oil, indicating that capsaicin and isothiocyanates excite nociceptors through distinct molecular mechanisms[18]. However, inflammatory responses to these irritants show partial cross-desensitization, suggesting that they act through convergent cellular signalling pathways[19,20].

To identify the cellular site of action of mustard oil, we used calcium imaging to ask whether allyl isothiocyanate (20 μM) excites dissociated sensory neurons from rat trigeminal ganglia. Approximately 35% of cultured neurons showed rapid and robust increases in intracellular free calcium with the application of this compound (Fig. 1). Subsequent exposure to capsaicin (1 μM) excited a larger cohort of neurons (~55%), encompassing all of the mustard-oil-responsive cells. Capsaicin-evoked responses result from an influx of divalent cations directly through the TRPV1 channel[21]. Similarly,

mustard-oil-evoked responses were eliminated when calcium was removed from the extracellular medium (not shown), consistent with a mechanism involving an influx of calcium across the plasma membrane.

The plant-derived cannabinoid receptor agonists THC and cannabinol have recently been shown to relax hepatic or mesenteric arteries *in vitro* by activating capsaicin-sensitive, CGRP-containing perivascular sensory nerve endings that innervate the smooth muscle[22]. This effect is not inhibited by antagonists of known G-protein-coupled cannabinoid receptors, but is blocked by ruthenium red, an inhibitor of TRPV1 and certain other members of the TRP channel family. Moreover, THC-evoked vasorelaxation is dependent on extracellular calcium and persists in TRPV1-deficient mice[22]. Like THC, isothiocyanates induce endothelium-independent vasorelaxation[23], and activation of sensory nerve endings by these compounds is blocked by ruthenium red[24] and persists in TRPV1$^{-/-}$ mice[18]. We therefore asked whether THC and allyl isothiocyanate excite the same population of neurons. Indeed, all THC-excitable cells responded to mustard oil (Fig. 1a, b), and responses to either agonist were blocked by ruthenium red (10 μM) (Fig. 1c). A significant percentage (~35%) of mustard-oil-sensitive neurons were not excited by THC, but most of these cells showed relatively weak responses to mustard oil (25% less than THC-sensitive cells, $P < 0.001$) and their failure to respond to THC may reflect the less robust effect of cannabinoids as excitatory agents for these cells (Fig. 1). Whole-cell patch-clamp recordings from trigeminal neurons confirmed our observation that a subpopulation of capsaicin-sensitive cells is dually responsive to mustard oil and THC (Fig. 1e). These agonists produced currents that reversed near 0 mV (consistent with the opening of non-selective cation channels), showed slight outward rectification and were blocked by ruthenium red (Fig. 1e).

Taken together, these results suggest that THC and mustard oil excite nociceptors through a similar, if not identical mechanism involving activation of a calcium-permeable, ruthenium-red-blockable channel on capsaicin-sensitive, CGRP-containing sensory neurons. In considering a potential molecular target for these agents, we realized that all of these characteristics are met by ANKTM1, a member of the TRP channel family recently proposed to function as a detector of noxious cold by peptidergic, TRPV1-expressing primary afferent neurons[4]. To test this possibility directly, we measured responses of rat ANKTM1-expressing human embryonic kidney (HEK) 293 cells to mustard oil. Indeed, application of mustard oil (20 μM) produced large increases in intracellular free calcium, whereas capsaicin (1 μM) had no effect (Fig. 2a). This response was specific to ANKTM1, as TRPV1- or

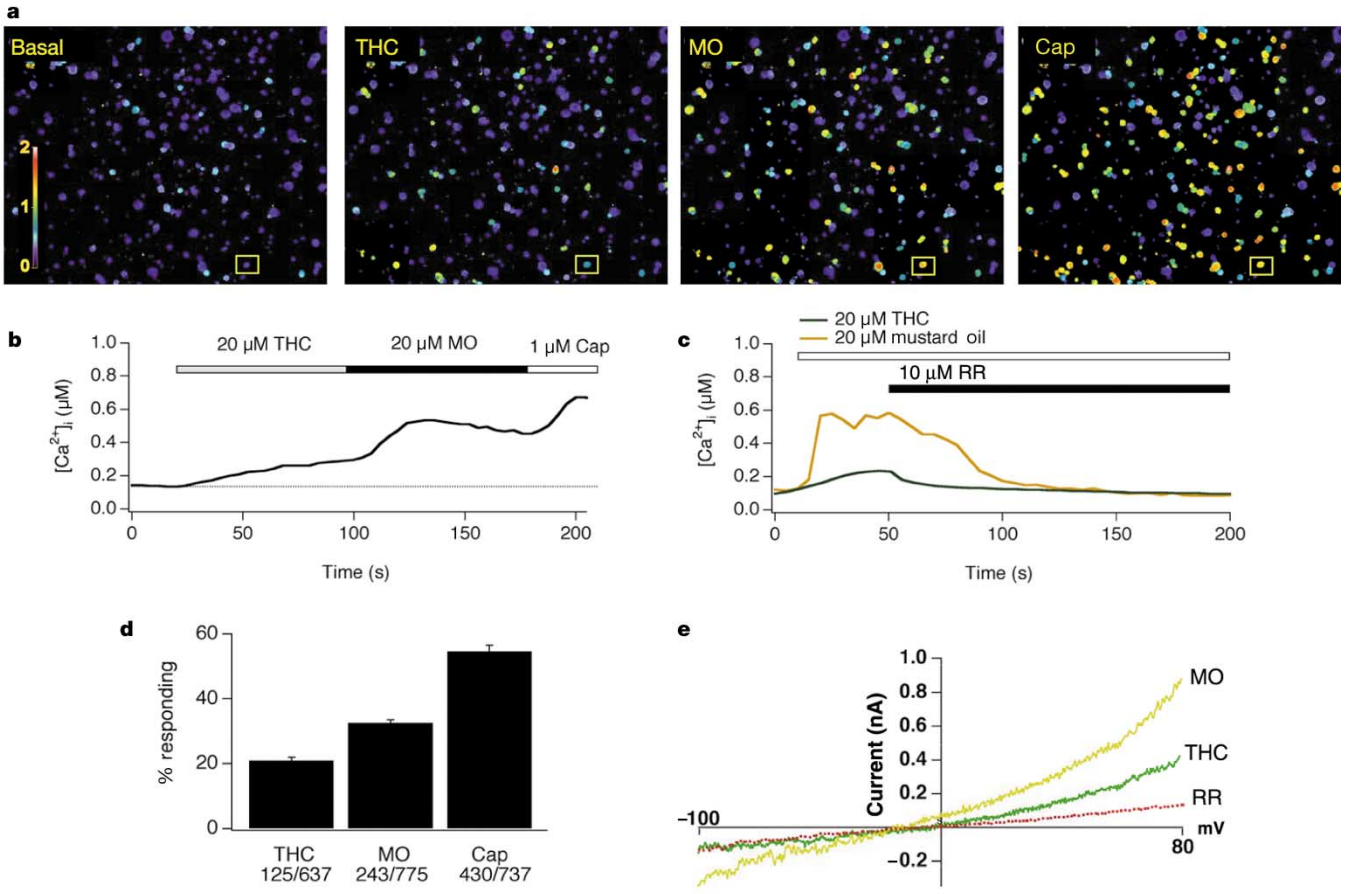

Figure 1 THC and mustard oil activate a subset of sensory neurons. **a**, Responses of dissociated rat trigeminal neurons to 20 μM THC, 20 μM mustard oil (MO) or 1 μM capsaicin (Cap), as measured by Fura-2 ratiometric imaging (scale bar indicates the intracellular calcium concentration, $[Ca^{2+}]_i$, in μM). **b**, $[Ca^{2+}]_i$ response in a representative neuron to THC, mustard oil and capsaicin (see box in part **a**). **c**, Calcium responses evoked by THC (green) and mustard oil (yellow) are blocked by 10 μM ruthenium red (RR). As shown in panel **b**, responses are sustained in the absence of ruthenium red. Calcium increases returned to basal levels when agonists were washed out (not shown). **d**, Percentage of cultured neurons exhibiting a stimulus-evoked rise in $[Ca^{2+}]_i$ in response to 20 μM THC, 20 μM mustard oil or 1 μM capsaicin. **e**, Electrophysiological response of a representative neuron to 20 μM THC (green) or 20 μM mustard oil in the absence (yellow) or presence (red) of 5 μM ruthenium red. Voltage ramps from −100 to +80 mV (200 ms) were applied every 2 s. THC-evoked currents display slight outward rectification. Mustard oil activates identical currents of larger amplitude in the same cell. Ruthenium red (10 μM) blocks THC- and mustard-oil-evoked currents.

vector-transfected cells were unresponsive. Mustard oil also elicited large and reversible membrane currents in oocytes expressing ANKTM1, where dose–response analysis revealed a half-maximal effective concentration (EC_{50}) of 11 ± 1 μM (Fig. 2b). To further verify the specificity of these ligands for ANKTM1, we tested a number of other TRP channels expressed by sensory neurons, including TRPV2, TRPV3 and TRPM8, none of which responded to mustard oil.

A variety of isothiocyanate compounds, including allyl, benzyl, phenylethyl, isopropyl and methyl isothiocyanate, constitute the main pungent ingredients in wasabi, yellow mustard, Brussels sprouts, nasturtium seeds and capers, respectively. Calcium imaging showed that each of these compounds was capable of activating the human ANKTM1 channel (Fig. 2c). Electrophysiological responses were also observed in oocytes expressing human ANKTM1, where large membrane currents were elicited in response to crude extracts prepared from brown mustard seed or wasabi paste (Fig. 2d), or to the isothiocyanates described above (Fig. 2e).

In view of our observation with trigeminal neurons, we asked whether cells heterologously expressing ANKTM1 also respond to THC or cannabinol. Calcium imaging showed that both the rat and the human receptor could be activated by these ligands (Fig. 3a, b), consistent with the idea that isothiocyanates and cannabinoids excite sensory neurons through the same molecular mechanism. Recordings from ANKTM1-expressing oocytes showed that cannabinoids elicit membrane currents that have similar, if not identical, properties to those produced by mustard oil (Fig. 3a, c), including slight outward rectification and block by ruthenium red (not shown). The synthetic cannabinoid receptor agonists HU-210 and CP55,940 do not produce neurogenic vasodilation[17] and failed to activate ANKTM1 (not shown). Finally, responses to cannabinoids were significantly smaller than those elicited by equal concentrations of isothiocyanates (Fig. 3c). Taken together, these characteristics closely resemble those observed in sensory neurons or sensory nerve fibres.

In situ hybridization studies indicate that only 4% of cells within mouse dorsal root ganglia express ANKTM1 transcripts[4], whereas we found that THC or mustard oil excites a larger population of neurons from rat trigeminal ganglia. This difference could reflect greater channel expression in rat trigeminal versus mouse dorsal root ganglia, upregulation of channel expression in culture and/or greater sensitivity of calcium imaging versus histological analysis. A significant part of this difference can be explained by the first of these possibilities, as *in situ* hybridization analysis that we carried out with trigeminal ganglia from newborn rats detected ANKTM1 expression in 20 ± 2% of neurons (644 of 3,221 cells) with a mean soma diameter of 18 ± 1 μm ($n = 88$). These numbers are consistent with our functional studies showing that mustard oil and THC excite ≥20% of cultured trigeminal neurons, encompassing 30–50% of small-diameter, capsaicin-sensitive cells. We therefore propose that mustard oils and THC mediate their excitatory effect on nociceptors through ANKTM1. Whether these compounds also act at other sites will be determined by the analysis of ANKTM1-deficient mice.

TRPV1 and TRPM8/CMR1 are thermosensitive channels that enable primary afferent neurons to detect heat and cold, respect-

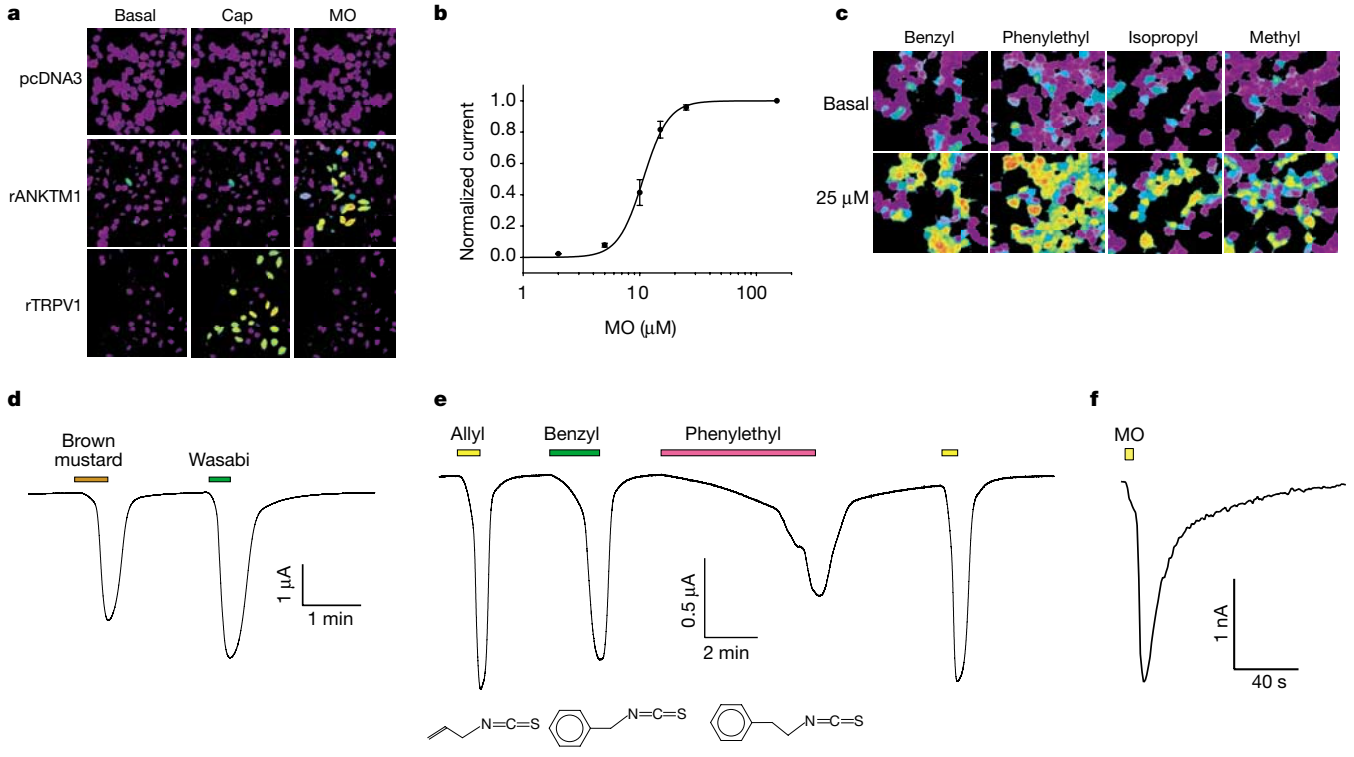

Figure 2 Activation of ANKTM1 by mustard oils in transfected mammalian cells and *Xenopus* oocytes. **a**, Mustard oil (allyl isothiocyanate, 20 μM) activates calcium influx into rat ANKTM1-expressing HEK293 cells (middle row), measured by Fura-2 fluorescence. Cells transfected with vector (pcDNA3, top) or TRPV1 (bottom) are insensitive to mustard oil. Only TRPV1-expressing cells are activated by capsaicin (1 μM). **b**, Dose–response curve for activation of rat ANKTM1 by mustard oil in oocytes (holding potential, $V_h = -40$ mV) recorded in nominally calcium-free ND96. Currents were normalized to maximal response evoked by 125 μM mustard oil. Half-maximal activation occurred at 11 ± 1 μM ($n = 5$). **c**, Activation of human ANKTM1 by different pungent mustard oils (benzyl-, phenylethyl-, isopropyl- and methyl isothiocyanate) in transfected HEK293 cells, measured by Fura-2 fluorescence. **d**, Activation of human ANKTM1 currents in oocytes by natural extracts of brown mustard and wasabi ($V_h = -60$ mV). **e**, Representative trace from an oocyte expressing human ANKTM1, showing activation by allyl-, benzyl- or phenylethyl isothiocyanate (10 μM each; $V_h = -60$ mV). Agonist structures are indicated below the current trace. **f**, Activation of human ANKTM1 by mustard oil in transfected HEK293 cells, as measured by whole-cell patch clamp ($V_h = -60$ mV). Brief application of mustard oil (50 μM for 3 s) activated large inward currents with a mean amplitude of 2.3 ± 0.3 nA ($n = 11$).

ively[12,25,26]. Activation of these receptors by plant-derived chemical agonists (capsaicin or menthol) produces sensations akin to those associated with the cognate thermal experiences. Recent studies suggest that ANKTM1 can be activated by thermal stimuli below 20 °C and may therefore serve as a detector of noxious cold[4]. Isothiocyanates produce a noxious sensation, as would be predicted for activation of a nociceptor, but the experience is qualitatively different from what one would expect if ANKTM1 simply served as a cold sensor. There are several possible explanations for this conundrum: (1) chemical and thermal agonists gate the channel and depolarize nociceptors differently to encode distinct psychophysical sensations; (2) isothiocyanates target several receptors, in addition to ANKTM1, to mediate their psychophysical effects; (3) activation of ANKTM1 alone produces a burning-like irritation, whereas the sensation of noxious cold requires the activation of additional channels, such as TRPM8; (4) a primary function of ANKTM1 may be to respond to chemical stimuli that produce pain and oedema, such as inflammatory peptides and neurotransmitters[27].

To address these possibilities, we carried out calcium imaging experiments with cultured rat trigeminal neurons to look for overlap between mustard oil and cold sensitivity. Among mustard-oil-sensitive neurons, 96% did not respond to a cold stimulus (5 °C). The 4% that were cold sensitive also responded to menthol, suggesting that activation of TRPM8 can account for cold sensitivity in these cells. When analysing neurons responding to cold, 95% responded to menthol and the remaining 5% were insensitive to both menthol and mustard oil. These results indicate that ANKTM1 is unlikely to underlie cold sensitivity in cultured trigeminal neurons, even in the small subpopulation of menthol-insensitive cells (Supplementary Fig. 1). We also examined the cold sensitivity of ANKTM1-expressing HEK293 cells and found no responses to 5 °C, even though they exhibited robust sensitivity to mustard oil. By contrast, TRPM8-expressing cells showed robust and reproducible responses to cold or menthol (Supplementary Fig. 1). In view of our inability to observe reliable responses of ANKTM1-expressing cells to cold, we asked whether the effects of mustard oil might be augmented by cold. Instead we found that cooling of the perfusate reduced mustard-oil-evoked currents in ANKTM1-expressing oocytes (Supplementary Fig. 2), as one might expect for effects of cold on a non-thermally responsive ion channel.

Many TRP channels are activated or modulated downstream of neurotransmitter or growth factor receptors that stimulate phospholipase C (PLC) pathways[14,16]. In considering alternative physiological roles for ANKTM1, we asked whether it might function as a receptor-operated channel in transfected HEK293 cells co-expressing the PLC-coupled M1 muscarinic acetylcholine receptor (mAChR). In cells expressing mAChR alone, activation by carbachol (100 μM) produced a modest rise in intracellular calcium due to release from intracellular stores (Fig. 4a). However, in cells co-expressing ANKTM1, carbachol application produced a much more sustained and robust increase in cytoplasmic calcium that could be blocked by ruthenium red (Fig. 4a). All cells showing this enhanced calcium response (40/100) were also activated by mustard oil (20 μM), whereas cells showing only the small release transient (60/100) were insensitive to mustard oil. Taken together, these results show that the large, sustained calcium increases were mediated through activation of ANKTM1. This was further supported by whole-cell patch-clamp recordings in which the application of acetylcholine (50 μM) produced rapidly developing inward currents in cells co-transfected with ANKTM1 and mAChR, but not in cells expressing mAChR alone (Fig. 4b).

Recent studies suggest that TRP channels can be regulated by one or more consequences of PLC activation, including phosphatidylinositol bisphosphate hydrolysis, the production of lipid metabolites or the release of calcium from intracellular stores[14,16]. To ask whether calcium release is sufficient to activate ANKTM1, we treated cells with thapsigargin to increase intracellular calcium in a receptor-independent manner. In vector-transfected control cells, thapsigargin (1 μM) induced a small and gradual increase in

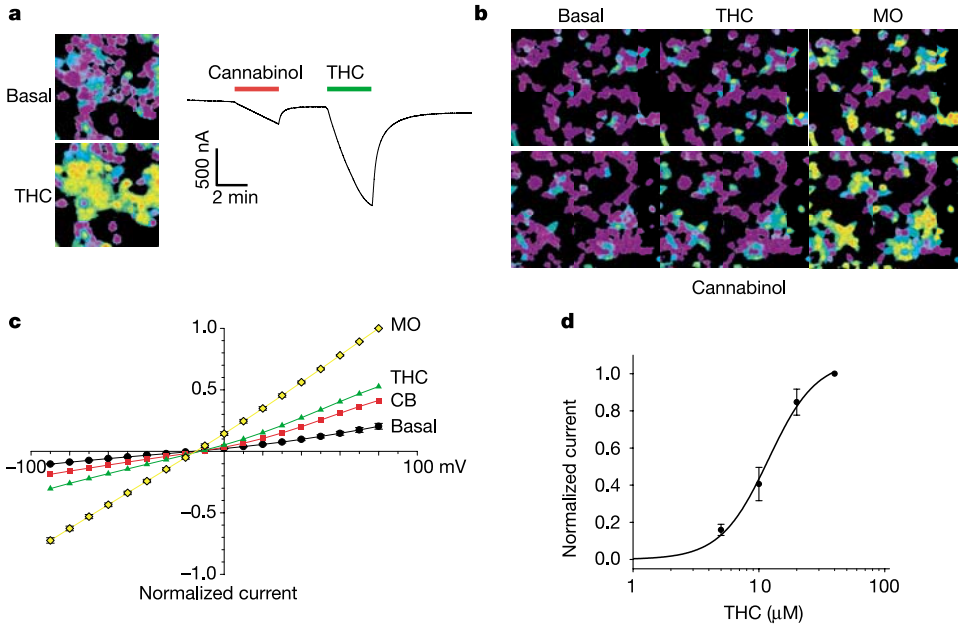

Figure 3 Activation of ANKTM1 by cannabinoids. **a**, Left, THC-induced calcium influx into HEK293 cells transfected with rat ANKTM1, as measured by Fura-2 fluorescence. Right, activation of inward currents at −60 mV by cannabinol (20 μM) or THC (20 μM) in an oocyte expressing rat ANKTM1. **b**, Activation of human ANKTM1 by THC (20 μM, top row), cannabinol (20 μM, bottom row) or mustard oil (20 μM) in transfected HEK293 cells. **c**, Oocytes expressing rat ANKTM1 were perfused with cannabinol (CB, red), THC (green) or mustard oil (yellow) (20 μM for 1 min each). Voltage ramps (−90 to +80 mV) were applied every 2 s. Averaged currents ($n = 5$ cells) were normalized to maximal mustard-oil-evoked responses at +80 mV. **d**, THC dose–response curve for human ANKTM1 in oocytes ($V_h = -60$ mV) recorded in nominally calcium-free ND96. Currents were normalized to maximal responses evoked by THC (40 μM). Half-maximal activation (EC_{50}) occurred at 12 ± 2 μM, $n_{Hill} = 2.3 \pm 0.4$ ($n = 3$). THC solubility is poor at concentrations exceeding 40 μM, thus preventing full saturation of the dose–response curve.

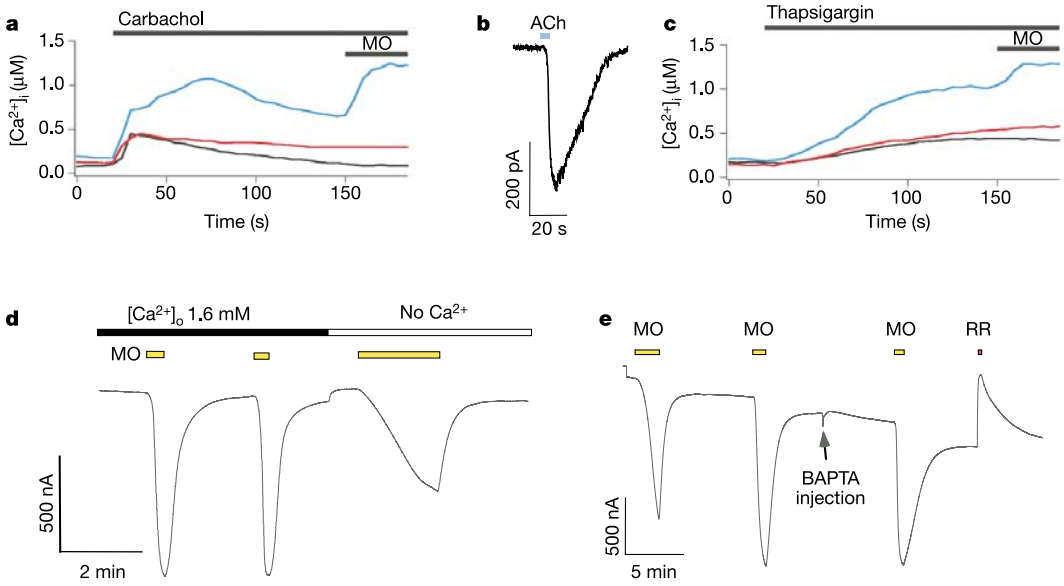

Figure 4 ANKTM1 is a receptor-operated channel. **a**, Application of carbachol (100 μM) to HEK293 cells expressing human ANKTM1 and mAChR evoked large $[Ca^{2+}]_i$ responses (blue trace). Co-application of ruthenium red (10 μM, red trace) reduced responses to levels observed in cells transfected with mAChR alone (black trace). ANKTM1 expression was assessed by sensitivity to mustard oil (20 μM). Traces represent averages of three experiments, 35–100 cells in each. **b**, Application of acetylcholine (ACh, 50 μM) produced inward currents in HEK293 cells expressing mAChR1 and human ANKTM1 ($V_h = -60$ mV). **c**, Application of thapsigargin (1 μM) was sufficient to elicit robust increases in $[Ca^{2+}]_i$ in ANKTM1-expressing HEK293 cells (blue trace). Co-application of thapsigargin and ruthenium red (10 μM, red trace) reduced responses to the level observed in vector (pcDNA3)-transfected control (black trace). **d**, Mustard-oil-evoked currents are augmented by extracellular calcium. An ANKTM1-expressing oocyte was activated twice by mustard oil (25 μM) in the presence of extracellular calcium ($[Ca^{2+}]_o = 1.6$ mM), and subsequently in calcium-free medium (0.5 mM EGTA). **e**, Activation of ANKTM1 by mustard oil does not require extracellular or intracellular calcium. Currents evoked by mustard oil (50 μM), measured in calcium-free medium, were identical before and after BAPTA injection (63 nl of a 120 mM solution was sufficient to eliminate receptor-mediated activation of calcium-dependent chloride current; see Supplementary Fig. 3). A slowly developing basal current could be blocked by ruthenium red (5 μM).

intracellular calcium (Fig. 4c). By contrast, cells expressing ANKTM1 showed large and rapid calcium increases that were blocked by ruthenium red (10 μM), suggesting that calcium release is sufficient to activate ANKTM1. We were therefore curious to know whether activation of ANKTM1 by THC or mustard oil is calcium dependent. We found that both the rate and the magnitude of responses to these agonists were enhanced in the presence of extracellular calcium (Fig. 4d). However, THC- or mustard-oil-evoked responses persisted even when both intracellular and extracellular calcium were chelated (Fig. 4e). This shows that calcium is not required for the activation of ANKTM1 by mustard oil or THC, but augments responses to these agonists through an as yet undetermined mechanism. Although these results are consistent with a model in which mustard oil and THC serve as direct channel agonists, they do not exclude an indirect activation mechanism requiring other cellular factors.

We have shown that plants of the genus *Brassica* (for example, wasabi, horseradish and mustard) and *Capsicum* (chilli peppers) use similar molecular strategies to produce irritation and inflammation in herbivorous mammals, both involving activation of excitatory TRP channels on primary sensory nerve endings. Our current findings also extend the concept that some TRP channels may serve as ionotropic cannabinoid receptors, which, in the context of the primary afferent nerve fibre, may contribute to inflammatory hypersensitivity or vasodilation[5–8,28]. Although THC and cannabinol are natural plant products, they are not known to function as endogenous transmitters in the vertebrate nervous system. In preliminary studies, we have not observed activation of ANKTM1 by known 'endocannabinoids', such as anandamide or *sn*-2-arachidonylglycerol (2-AG), but our findings raise the possibility that other structurally related lipid second messengers may excite nociceptors by targeting this member of the TRP channel family. Finally, our data suggest that ANKTM1 may also serve to excite nociceptors in response to one or more pro-algesic or pro-inflammatory agents—for example, bradykinin, histamine, serotonin, ATP and neurotrophins—that activate PLC signalling pathways in these cells[27]. Future genetic and pharmacological studies will determine the extent to which this channel contributes to thermal or chemical nociception. □

Methods

Neuronal cell culture, calcium imaging and patch-clamp recording

Trigeminal ganglia from newborn (postnatal day 0) Sprague–Dawley rats were cultured as previously described[25]. Calcium imaging using Fura-2/AM (Molecular Probes) was analysed using automated routines written in Igor Pro (Wavemetrics). For electrophysiology, gigaseals were formed with pipettes (WPI, Inc.) that had a resistance of 3–5 MΩ in standard pipette solution. Liquid junction potentials (measured in separate experiments) did not exceed 3 mV and thus no correction for this offset was made. Whole-cell voltage clamp was performed at a holding potential of −20 mV with a 200 ms voltage ramp from −100 mV to +80 mV at 3.6 Hz. Data were acquired using Pulse and Pulsefit (HEKA GmbH) software. Recordings were sampled at 20 kHz and filtered at 2 kHz. Extracellular Ringer's solution contained (in mM): 155 NaCl, 4.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose and 5 HEPES-Na (pH 7.4). For calcium-free solution, 2 mM $MgCl_2$ and 1 mM EGTA were substituted for $CaCl_2$. For mustard oil and muscarinic activation of ANKTM1 in HEK293 cells, recording solutions were as follows (in mM): external, 140 NaCl, 1 $MgCl_2$, 1 $CaCl_2$, 1 EGTA and 10 HEPES-Na (pH 7.4); internal, 140 NaCl, 1 $MgCl_2$, 10 HEPES-Na (pH 7.2), 1 EGTA and 0.2 NaGTP.

ANKTM1 cloning and *in situ* hybridization histochemistry

Rat ANKTM1 sequence was identified by BLAST align of the ENSEMBL and GenBank rat genomic databases with the published mouse sequence (XM_232586.1 and contig RNOR01102533). Full-length complementary DNA (GenBank accession number AY496961) was cloned by polymerase chain reaction (PCR) from an adult rat trigeminal cDNA library using oligonucleotides ATG AAG CGC AGC TTG AGG AGG GTT CTG (5′) and CTA GAT GTC TGG GTG GCT AAT AGA ACA ATG TG (3′) and KOD polymerase (Novagen-Toboyo). A human ANKTM1 cDNA was kindly provided by B. Trueb[3]. Apparent mismatches with the ENSEMBL genomic sequence were corrected by PCR and the final product cloned into the pFROG3 mammalian/oocyte expression vector[29]. *In situ* hybridization histochemistry was performed as previously described[12] using probes corresponding to nucleotides 569–3,378 of the rat ANKTM1 sequence.

Expression in HEK293 cells and oocytes

HEK293 cells were plated on polylysine-coated coverglass or Lab-Tek II CC2 chamberslides (Nalgene-Nunc). Cells were transfected with Lipofectamine 2000 (Invitrogen) using 5–25 ng rat or human ANKTM1 plasmid per cm^2; pcDNA3 vector was added to bring the total amount of plasmid DNA to 100 ng cm^{-2}. For muscarinic activation, 100 ng cm^{-2} human mAChR plasmid was co-transfected with 10 ng cm^{-2} ANKTM1 plasmid. Sixteen hours after transfection, cells were loaded with Fura-2AM (5 μM) for 30 min and imaged in Ringer's solution. For oocyte expression, constructs were linearized with *Mlu*I and transcribed with T7 polymerase (Ambion). Oocytes were incubated in ND96 containing 5 μM ruthenium red as described[4]. Currents were recorded in ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES pH 7.6) or calcium-free ND96 (containing 100 μM $BaCl_2$) as indicated.

Preparation of natural extracts

For brown mustard, 3 g of ground brown mustard seeds were suspended in 10 ml ND96 and incubated for 2 h at room temperature. Extract was cleared by centrifugation at 3,000*g* for 20 min. The supernatant fluid was filtered through a 0.2 μm filter and diluted 20-fold in ND96. For wasabi, 0.5 g of 100% pure wasabi paste (Pacific Farms, Oregon) was suspended in 1 ml ND96, clarified by centrifugation at 18,000*g*, filtered and diluted 50-fold in ND96.

Received 7 November; accepted 12 December 2003; doi:10.1038/nature02282.
Published online 7 January 2004.

1. Wall, P. D. & Melzack, R. *The Textbook of Pain* (W. B. Saunders, London, 1999).
2. Jancso, N., Jancso-Gabor, A. & Szolcsanyi, J. Direct evidence for neurogenic inflammation and its prevention by denervation and by pretreatment with capsaicin. *Br. J. Pharmacol.* **31,** 138–151 (1967).
3. Jaquemar, D., Schenker, T. & Trueb, B. An ankyrin-like protein with transmembrane domains is specifically lost after oncogenic transformation of human fibroblasts. *J. Biol. Chem.* **274,** 7325–7333 (1999).
4. Story, G. M. *et al.* ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell* **112,** 819–829 (2003).
5. Zygmunt, P. M. *et al.* Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature* **400,** 452–457 (1999).
6. Bisogno, T. *et al.* Molecular targets for cannabidiol and its synthetic analogues: effect on vanilloid VR1 receptors and on the cellular uptake and enzymatic hydrolysis of anandamide. *Br. J. Pharmacol.* **134,** 845–852 (2001).
7. Di Marzo, V., Bisogno, T. & De Petrocellis, L. Anandamide: some like it hot. *Trends Pharmacol. Sci.* **22,** 346–349 (2001).
8. Ross, R. A. Anandamide and vanilloid TRPV1 receptors. *Br. J. Pharmacol.* **140,** 790–801 (2003).
9. Lembeck, F. & Holzer, P. Substance P as neurogenic mediator of antidromic vasodilation and neurogenic plasma extravasation. *Naunyn Schmiedebergs Arch. Pharmacol.* **310,** 175–183 (1979).
10. Louis, S. M., Jamieson, A., Russell, N. J. & Dockray, G. J. The role of substance P and calcitonin gene-related peptide in neurogenic plasma extravasation and vasodilatation in the rat. *Neuroscience* **32,** 581–586 (1989).
11. Szallasi, A. & Blumberg, P. M. Vanilloid (capsaicin) receptors and mechanisms. *Pharmacol. Rev.* **51,** 159–212 (1999).
12. Caterina, M. J. *et al.* The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* **389,** 816–824 (1997).
13. Tominaga, M. *et al.* The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron* **21,** 531–543 (1998).
14. Montell, C., Birnbaumer, L. & Flockerzi, V. The TRP channels, a remarkably functional family. *Cell* **108,** 595–598 (2002).
15. Jordt, S. E., McKemy, D. D. & Julius, D. Lessons from peppers and peppermint: the molecular logic of thermosensation. *Curr. Opin. Neurobiol.* **13,** 487–492 (2003).
16. Clapham, D. E. TRP channels as cellular sensors. *Nature* **426,** 517–524 (2003).
17. Reeh, P. W., Kocher, L. & Jung, S. Does neurogenic inflammation alter the sensitivity of unmyelinated nociceptors in the rat? *Brain Res.* **384,** 42–50 (1986).
18. Caterina, M. J. *et al.* Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science* **288,** 306–313 (2000).
19. Jancso, G., Kiraly, E. & Jancso-Gabor, A. Pharmacologically induced selective degeneration of chemosensitive primary sensory neurones. *Nature* **270,** 741–743 (1977).
20. Simons, C. T., Carstens, M. I. & Carstens, E. Oral irritation by mustard oil: self-desensitization and cross-desensitization with capsaicin. *Chem. Senses* **28,** 459–465 (2003).
21. Caterina, M. J. & Julius, D. The vanilloid receptor: a molecular gateway to the pain pathway. *Annu. Rev. Neurosci.* **24,** 487–517 (2001).
22. Zygmunt, P. M., Andersson, D. A. & Hogestatt, E. D. Δ^9-Tetrahydrocannabinol and cannabinol activate capsaicin-sensitive sensory nerves via a CB1 and CB2 cannabinoid receptor-independent mechanism. *J. Neurosci.* **22,** 4720–4727 (2002).
23. Wilson, R. K., Kwan, T. K., Kwan, C. Y. & Sorger, G. J. Effects of papaya seed extract and benzyl isothiocyanate on vascular contraction. *Life Sci.* **71,** 497–507 (2002).
24. Patacchini, R., Maggi, C. A. & Meli, A. Capsaicin-like activity of some natural pungent substances on peripheral endings of visceral primary afferents. *Naunyn Schmiedebergs Arch. Pharmacol.* **342,** 72–77 (1990).
25. McKemy, D. D., Neuhausser, W. M. & Julius, D. Identification of a cold receptor reveals a general role for TRP channels in thermosensation. *Nature* **416,** 52–58 (2002).
26. Peier, A. M. *et al.* A TRP channel that senses cold stimuli and menthol. *Cell* **108,** 705–715 (2002).
27. Rang, H. P., Bevan, S. & Dray, A. in *Textbook of Pain* (eds Wall, P. D. & Melzack, R.) 57–78 (Churchill-Livingstone, London, 1994).
28. Smart, D. *et al.* The endogenous lipid anandamide is a full agonist at the human vanilloid receptor (hVR1). *Br. J. Pharmacol.* **129,** 227–230 (2000).
29. Jordt, S. E., Tominaga, M. & Julius, D. Acid potentiation of the capsaicin receptor determined by a key extracellular site. *Proc. Natl Acad. Sci. USA* **97,** 8134–8139 (2000).

Supplementary Information accompanies the paper on **www.nature.com/nature**.

Acknowledgements We are grateful to G. Hollopeter for generation of the adult rat trigeminal cDNA library and to B. Trueb for providing us with human ANKTM1 cDNA. This work was supported by grants from the Segerfalk Foundation and the Swedish Research Council (P.Z. and E.H.), the American Heart Association (H.C.) and the National Institutes of Health (I.M., D.B. and D.J.).

Competing interests statement The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to D.J. (julius@cmp.ucsf.edu).

erratum

Structure and conserved RNA binding of the PAZ domain

Kelley S. Yan, Sherry Yan, Amjad Farooq, Arnold Han, Lei Zeng & Ming-Ming Zhou

Nature **426,** 469–474 (2003).

In this Letter, the last two sentences of the second paragraph of the left column on page 470 should read: "Preliminary data using an immobilized 5′ phosphorylated ssRNA with a 3′-biotin modification showed that this RNA failed to pull down purified Ago1 PAZ domain (data not shown). By NMR titration, this RNA had a reduced binding affinity (K_d greater than 20 μM), suggesting that the 3′ end may also play a role in PAZ domain interaction." □

corrigendum

Eya protein phosphatase activity regulates Six1–Dach–Eya transcriptional effects in mammalian organogenesis

Xue Li, Kenneth A. Ohgi, Jie Zhang, Anna Krones, Kevin T. Bush, Christopher K. Glass, Sanjay K. Nigam, Aneel K. Aggarwal, Richard Maas, David W. Rose & Michael G. Rosenfeld

Nature **426,** 247–254 (2003).

In this Article, K. A. Ohgi's surname was misspelled. It is presented correctly here and has been amended in the HTML version of the paper on *Nature*'s website (http://www.nature.com/nature/). □