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**Abstract**

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**SUMMARY**

Specialized somatosensory neurons detect temperatures ranging from pleasantly cool or warm to burning hot and painful (nociceptive). The precise temperature ranges sensed by thermally sensitive neurons is determined by tissue-specific expression of ion channels of the transient receptor potential (TRP) family. We show here that in *Drosophila*, TRPA1 is required for the sensing of nociceptive heat. We identify two previously unidentified protein isoforms of dTRPA1, named dTRPA1-C and dTRPA1-D, that explain this requirement. A dTRPA1-C/D reporter was exclusively expressed in nociceptors, and dTRPA1-C rescued thermal nociception phenotypes when restored to mutant nociceptors. However, surprisingly, we find that dTRPA1-C is not a direct heat sensor. Alternative splicing generates at least four isoforms of dTRPA1. Our analysis of these isoforms reveals a 37-amino-acid-long intracellular region (encoded by a single exon) that is critical for dTRPA1 temperature responses. The identification of these amino acids opens the door to a biophysical understanding of a molecular thermosensor.

**INTRODUCTION**

Nociception is the sensorineural process of encoding noxious stimuli. The ability to sense and avoid potential or actual tissue-damaging stimuli, such as noxious temperature, mechanical stimuli, and irritant chemicals, is critical for survival. Transient receptor potential (TRP) channels have been shown to play an important role in a variety of sensory systems. Several members of this family have been shown to be involved in nociception (Bautista et al., 2006; Caterina et al., 2000; Kwan et al., 2006).

In mammals, TRPA1 has been implicated as a key player in nociception. The recent identification of a mutation in TRPA1 that is associated with a heritable familial episodic pain syndrome (FEPS) in a Colombian family represents the first human pain-related syndrome to be linked to the TRP gene superfamily (Kremeyer et al., 2010; Waxman, 2010). In mice, TRPA1 is detected in a subset of TRPV1-expressing dorsal root ganglion (DRG) C fibers and Aδ fibers, which are the nociceptive afferents. TRPA1 has been found to be required for chemical, mechanical, and noxious cold nociception, although the latter remains controversial (Bautista et al., 2006; Brierley et al., 2011; Jordt et al., 2004; Kwan et al., 2006; Story et al., 2003).

Two recent studies have now shown that *Drosophila* TrpA1 (dTrpA1) is required for thermal and mechanical nociception (Babcock et al., 2011; Neely et al., 2011) in both larvae and in adult flies. The findings of these studies are surprising because the temperature threshold of the dTRPA1 thermoTRP channel (27°C) does not match the temperature threshold for baseline nociception (39°C). In addition, dTrpA1 reporters are not expressed in nociceptive neurons, which leaves the site of action for dTrpA1 in nociception pathways unclear.

Here, using a newly isolated null mutant allele of dTrpA1, we further demonstrate that dTrpA1 indeed plays a role in thermal and mechanical nociception. We have identified transcripts encoding previously unknown isoforms of dTRPA1 (named dTRPA1-C and dTRPA1-D) that have biophysical properties distinct from the canonical dTRPA1-A isoform. A transgenic reporter for dTrpA1-C and dTrpA1-D is specifically expressed in the nociceptors of *Drosophila* larvae. Expression of the dTRPA1-C isoform in heterologous cells suggests that it is not a direct temperature sensor at temperatures as high as 42°C, but it does respond to isothiocyanate compounds. Nevertheless,
**Figure 1.** *dTrpA1* Is Required for Thermal and Mechanical Nociception

(A) Schematic diagram of *dTRPA1* protein. Green circles indicate ankyrin repeats, while blue ovals indicate transmembrane helices. Asterisk indicates location of the premature stop codon in *dTrpA1W903*.

(B–F) The distribution of NEL latency for wandering third-instar larvae stimulated with a 46°C probe. (B) The distribution of thermal nociception responses of wild-type Canton S larvae (4 trials, n = 115). (C) The distribution of thermal nociception responses of *dTrpA1W903*/+ larvae (3 trials, n = 80) resembled wild-type Canton S (Wilcoxon rank-sum test with Bonferroni correction, p > 0.9). (D) The latencies for thermal nociception responses of *Df(3L)ED4415/+* larvae (3 trials, n = 54) are slightly delayed in comparison to wild-type (Wilcoxon rank-sum test with Bonferroni correction, p < 0.01). (E) *dTrpA1W903* mutant larvae (4 trials, n = 149) showed severely delayed nociception responses in comparison to control strains Canton S and *dTrpA1W903*/+ (Wilcoxon rank-sum test with Bonferroni correction, p < 0.0001). (F) *dTrpA1W903*/+/*Df(3L)ED4415* larvae (3 trials, n = 67) showed delayed NEL in comparison to control strains Canton S, *dTrpA1W903*/+ and *Df(3L)ED4415/+*, indicating that *Df(3L)ED4415* failed to complement the NEL defects of *dTrpA1W903* (Wilcoxon rank-sum test with Bonferroni correction, p < 0.0001).

(G) *dTrpA1* is required for mechanical nociception. A significantly reduced proportion of *dTrpA1W903* [3 trials, n = 97, Pearson’s chi-square test for independence with Bonferroni correction, p < 0.001(***)] in comparison to Canton S or *dTrpA1W903*/+ and *dTrpA1W903*/+/*Df(3L)ED4415* [3 trials, n = 80 Pearson’s chi-square test
expression of dTrpA1-C in nociceptors rescued thermal nociception phenotypes of dTrpA1 mutants. Our results suggest a role for dTRPA1 in thermal nociception that does not depend on thermosensitivity. Furthermore, analysis of the four existing dTRPA1 isoforms reveals 37 intracellular amino acids (between the last ankyrin repeat and membrane-spanning segment S1) as playing a critical role temperature sensing. Sequences at the absolute N terminus also affect temperature responses. The identification of these heat-responsive elements, which we term TRP Ankyrin Caps (TACs), will enable a biophysical understanding of heat sensing by TRPA1 channels.

RESULTS AND DISCUSSION

Identification of a Chemically Induced dTrpA1 Null Mutant Allele

We performed a screen for ethyl methanesulphonate (EMS)-induced mutations of the dTrpA1 locus through the Drosophila Tilling Project (Cooper et al., 2008). One line stood out as a potential null loss-of-function allele of dTrpA1. As in all TRP channels, dTRPA1 is predicted to have six transmembrane domains, with a pore loop between the fifth and sixth transmembrane domain. In the EMS-induced mutant allele dTrpA1W903*, a guanosine is mutated to an adenosine (Figure S1 available online), changing the codon for Tryptophan 903 to a premature amber stop codon. As this residue was located upstream of the pore loop, the premature stop codon located in the fourth transmembrane domain of dTrpA1 was predicted to lead to nonsense-mediated decay or the production of a nonfunctional dTRPA1 channel from all possible dTRPA1 transcripts (Figure 1A). In addition, Df(3L)ED4415, a genomic deletion (deficiency) removing 210 kb that included dTrpA1 (as well as 25 other genes), was available for our studies. As expected for a large deficiency, Df(3L)ED4415 is not homozygous viable. In order to separate the dTrpA1W903* mutation from other unlinked EMS-induced mutations that might be present in the mutagenized strain, we outcrossed the dTrpA1W903* mutant to Df(3L)ED4415 (Figure 1F). The average summed gentle touch response scores of wild-type Canton S (n = 28) and dTrpA1 (3 trials, n = 67) animals. (H) The average summed gentle touch response scores of wild-type Canton S (n = 28) and dTrpA1 mutants dTrpA1W903* (n = 28), dTrpA1W903*/+ (n = 16) and dTrpA1W903*/Df(3L)ED4415 (n = 21) were not significantly different (single-factor ANOVA, p = 0.13).

RNAi Knockdown of dTrpA1 Expression in mdIV Neurons

Phenocopies the Mutant

The class IV multidendritic (mdIV) neurons function as polymodal nociceptors in Drosophila larvae (Hwang et al., 2007). These neurons are known to respond in extracellular recordings toAITC in a dTRPA1-dependent manner (Xiang et al., 2010). Yet, previously existing dTrpA1 reporters are not expressed in these cells (Hamada et al., 2008; Rosenzweig et al., 2005) (W.D.T. and L.Z., unpublished data). Thus, to test whether dTrpA1 was required in the nociceptors for mechanical and thermal nociception, we used the nociceptors-specific pickpocket1.9-GAL4 (ppk-GAL4) driver to express UAS-dTrpA1-RNAi and UAS-dicer2 (Hwang et al., 2007). The ppk-GAL4/+; UAS-dTrpA1RNAi/UAS-dicer2 larvae showed reduced responses to both noxious thermal and mechanical stimuli (Figures 2D and 2F). Although RNAI mutant phenotypes were less severe than those of the null mutant, these results support the involvement of dTRPA1 in nociception and further suggest that the site of action for dTrpA1 is in the nociceptors themselves.

Properties of the Known Isoforms of dTrpA1 Are Not Consistent with a Function in Nociception

The heat activation threshold of the known heat-sensing dTRPA1-A ion channel is between 24°C and 29°C (Viswanath et al., 2003). In contrast, both the behavioral threshold for larval nociception and the activation threshold of the mdIV neurons are

for independence with Bonferroni correction, p < 0.001(*) in comparison to Canton S and Df(3L)ED4415/+ or dTrpA1W903*+/+ larvae showed NEL responses to 30 mM von Frey fibers relative to wild-type Canton S larvae (5 trials, n = 120), dTrpA1W903*+/ heterozygous (3 trials, n = 63), and Df(3L)ED4415/+ heterozygous (3 trials, n = 67) animals.

(H) The average summed gentle touch response scores of wild-type Canton S (n = 28) and dTrpA1 mutants dTrpA1W903* (n = 28), dTrpA1W903*/+ (n = 16) and dTrpA1W903*/Df(3L)ED4415 (n = 21) were not significantly different (single-factor ANOVA, p = 0.13).

In (B–H), error bars indicate the SEM.
approximately 39°C (Tracey et al., 2003; Xiang et al., 2010). Thus, although our behavioral results, as well as the recent results of others (Babcock et al., 2011), suggested a site of action for dTrpA1 in nociceptors, the known biophysical properties of dTRPA1-A seemed inconsistent with this possibility.

If dTRPA1-A was expressed in nociceptors, then the predicted behavioral threshold for nociception would be 29°C. Indeed, consistent with this prediction, larvae with forced expression of the dTrpA1-A isoform in the nociceptor neurons (ppk-GAL4/+; UAS-dTrpA1RNAi) had a dramatically lowered thermal nociception threshold. Greater than ninety percent of these larvae responded to a 30°C heat stimulus with NEL in less than 1 sec (Figure 3). This was in dramatic contrast to the behavior of wild-type larvae and other control genotypes, which never
produced NEL in response to 30°C heat (Figure 3). The behavior of larvae expressing dTRPA1-A in nociceptors causes thermal allostynia that is even more severe, and with a lower threshold (<30°C), than that which is seen following exposure of larvae to tissue-damaging UV-C radiation (which causes a behavioral thermal nociception threshold of 34°C [Babcock et al., 2009]).

Several important conclusions can be drawn from these results. First, the results further confirmed that mdIV neurons are indeed nociceptors, since expression of dTRPA1-A in mdIV neurons altered the thermal threshold of NEL behavior in an intuitively predictable manner. Second, the results argued against a role for dTRPA1-A in mediating baseline nociception in the mdIV neurons. This is in apparent conflict with the results of dTRPA1 mutant behavior, genomic rescue, and RNAi knockdown experiments, which strongly suggest that dTRPA1 is required in mdIV neurons for nociception. Furthermore, since existing dTRPA1 reporters are not expressed in mdIV neurons, another known isoform of dTRPA1 (dTRPA1-B; Figure 4B), which shares a transcription start site with dTRPA1-A (Kwon et al., 2010), could not explain the requirement for dTRPA1 in nociception behaviors.

**Cloning of dTRPA1 Isoforms**

A potential explanation for these findings was found when we examined the genomic region surrounding the dTRPA1 locus and identified two highly conserved putative exons that were located upstream of the known transcriptional start site for dTRPA1-A and dTRPA1-B. These exons were historically annotated as part of the misfire (mfr) gene (W.D.T. and L.Z., unpublished data), and this may have caused them to be unnoticed in earlier studies. Current annotations predict that these exons may be part of the dTRPA1 locus and spliced into the first exon of dTRPA1-A/B. To test the possibility that these newly identified exons were indeed part of the dTRPA1 locus, we performed RT-PCR. We successfully amplified, cloned, and sequenced PCR products from these reactions, identifying two previously unidentified dTRPA1 transcripts (Figures S2A and S2B). To distinguish these transcripts from the known transcripts of dTRPA1 (dTRPA1-A and dTRPA1-B) (Figures 4A and 4B), we refer to these transcripts as dTRPA1-C and dTRPA1-D (Figures 4C and 4D).

These transcripts are distinct from the dTRPA1-A and dTRPA1-B transcripts. They are also distinct from other dTRPA1 transcripts that have been predicted to exist by FlyBase, the Drosophila genome project (http://flybase.org/cgi-bin/gbrowse/dmel/?Search=1;name=FBgn0035934), because they do not include the third exon of the annotated dTRPA1 locus (Figures 4A–4D). The DNA sequence of plasmid clones of dTRPA1-C and dTRPA1-D confirmed the incorporation of the two upstream exons into dTRPA1 transcriptional start and splice variants. Alternative splicing of dTRPA1-C and dTRPA1-D results in skipping of the initial exon (the third exon of the locus) that is used in dTRPA1-A/B transcripts (Figures 4C and 4D). In contrast to the genome annotation, direct sequencing of PCR products used in cloning experiments indicated that the third exon of the locus was not spliced into the first or second exons at detectable levels (data not shown).

In addition, two alternatively spliced downstream exons exist in the four transcripts. The 12th exon of dTRPA1 is shared in the dTRPA1-A and dTRPA1-D transcripts (Figures 4A and 4D), and the 13th exon of the locus is shared between dTRPA1-B and dTRPA1-C (Figures 4B and 4C).

Four distinct proteins are predicted from these transcripts. At the dTRPA1-C/D N-termini, 97 amino acids are encoded by exons 1 and 2, and these amino acids do not share sequence similarity with the first 62 amino acids that are encoded by the first exon of dTRPA1-A/B (Figure S2C). In addition, the alternatively spliced 13th exon that is shared between dTRPA1-B and dTRPA1-C encodes 36 amino acids that are not easily aligned with 37 amino acids that are encoded by the shared exon 12 of dTRPA1-A and dTRPA1-D (Figure S2D). It is important to note that the amino acid variants of the protein do not change ankyrin repeats per se. Rather, the alternate amino acids flank the ankyrin repeats at the very N terminus and immediately following the last ankyrin repeat (Figures 4C and 4D).

**nociceptive Neurons Express dTRPA1-C and Require It for Nociception**

As noted above, existing GAL4 reporter strains for dTRPA1-A/B (Hamada et al., 2008; Rosenzweig et al., 2005; Tian et al., 2009) are not expressed in the mdIV nociceptive neurons (W.D.T. and L.Z., unpublished data). However, these reporters were made through the utilization of genomic DNA from regions largely downstream of the transcriptional start site of dTRPA1-C/D, and it is thus likely that they lack important upstream enhancer elements for dTRPA1-C/D (Figure 4B). Therefore, to investigate the expression pattern of dTRPA1-A-C/D, we cloned the 2.1 kb genomic interval between the transcriptional start site of dTRPA1-C/D and the 3’ end of misfire into a GAL4 reporter...
Figure 4. Gene and Protein Structures of Four Isoforms of dTrpA1 and the dTrpA1-C/D Expression Pattern
(A) Gene and protein structures of the canonical warmth-activated dTrpA1-A isoform. The dTRPA1-A first exon and alternatively spliced 12th exon are labeled in red. The blue bar located above the gene illustrates the region of DNA sequence used for making a previously described dTrpA1-A-GAL4 reporter (Hamada et al., 2008). The dTRPA1-A protein is predicted to have six transmembrane domains with 13 ankyrin repeats at the N terminus (Viswanath et al., 2003). The regions marked in red on the protein schematic are encoded by the red-labeled exons in the gene structure diagram.
(B) Gene and protein structures of the dTrpA1-B isoform. The dTrpA1-B isoform uses the first exon of dTrpA1-A (red) and is alternatively spliced to include the 13th exon (black). The red- and black-labeled regions flanking the ankyrin repeats in the protein schematic are encoded by the red exon 3 and black exon 13 in the gene structure diagram.
(C) Isoform dTrpA1-C, which uses a newly identified start site to include two 5’ exons marked in black (exons 1 and 2) and is alternatively spliced to include the 13th exon (black). The blue bar below the gene illustrates the intergenic region between the neighboring gene mfr and the dTrpA1-C isoform used for making the dTrpA1-C/D-GAL4 reporter. The black-labeled regions flanking the ankyrin repeats in the protein schematic are encoded by the black exons in the gene structure diagram.
(D) Gene and protein structures of the dTrpA1-D isoform. The dTrpA1-D isoform uses the exons 1 and 2 (black) and is alternatively spliced to include the 12th exon (red). The red- and black-labeled regions flanking the ankyrin repeats in the protein schematic are encoded by the black exons 1 and 2 and red exon 12 in the gene structure diagram.
(E) dTrpA1-C/D-GAL4 showed specific expression in larval mdIV neurons. The image is a maximum-intensity projection of a dorsal mdIV neuron (ddaC) (dTrpA1-C/D-GAL4:UAS-mCD8GFP, third instar).
transformation vector and generated transgenic GAL4 reporter fly strains (Figure 4B). Remarkably, dTrpA1C/D-GAL4 driving expression of UAS-mCD8GFP showed nearly exclusive mCD8GFP expression in the larval mdIV nociceptors and their central projections (Figures 4E, 4F, and 4G). This expression pattern led us to further hypothesize that either dTRPA1-C or dTRPA1-D might be the functional isoform of the mdIV nociceptors. Since the apparent activation temperature of a heterologously expressed dTRPA1-D isoform (34°C; see below) did not match the baseline thermal nociception threshold of larval nociceptors (39°C), we focused on the possibility that dTRPA1-C was involved in thermal nociception.

In order to directly test the hypothesis that dTRPA1-C was the isoform required for nociception, we generated UAS-dTrpA1-C transgenic flies and performed tissue-specific rescue experiments. Driving expression of UAS-dTrpA1-C specifically in the nociceptors (under control of ppk-GAL4) rescued the thermal nociception phenotypes of dTRPA1W903*/Df(3L)ED4415 mutant animals (Figure 5). In addition, in contrast to UAS-dTrpA1-A, expression of UAS-dTrpA1-C did not lower the behavioral threshold for thermal nociception (Figure 3). Combined, these results demonstrate that dTRPA1-C is the important dTRPA1 isoform required for nociception, and that it is required in nociceptors. Furthermore, these results constitute formal genetic proof that the mutation in dTrpA1 is responsible for the noxious heat-insensitive phenotype in the dTRPA1W903* mutant strain.

Interestingly, although the dTrpA1 genomic transgene did completely rescue mechanical nociception phenotypes (Figure 2E), dTRPA1-C was not sufficient to rescue mechanical nociception of dTrpA1 mutants when restored to mdIV neurons (L.Z. and W.D.T., unpublished data). This suggests that a specific complement of dTRPA1 isoforms is needed for mechanical nociception. It is possible that dTRPA1-D could be needed for mechanical nociception, or an as-yet-unidentified isoform could be involved.

**TAC Elements Confer Distinct Temperature-Sensing Properties to dTRPA1 Isoforms**

The observation that expression of dTRPA1-A or dTRPA1-C in the mdIV neurons conferred distinct temperature thresholds for the induction of NEL suggested that these two isoforms might have differing biophysical properties. These functional differences may be determined by the amino acids encoded by the alternatively spliced exons. The N-terminal region of dTRPA1 contains a relatively long string of ankyrin repeats that play an unknown role in determining the functional properties of the channel. The alternate amino acids of dTRPA1-A, dTRPA1-B, dTRPA1-C, and dTRPA1-D encode amino acids that flank these repeats (Figures 4A–4D). As mentioned above, we term these amino acid sequences TACs in order to distinguish the isoform-specific sequences from other regions of the dTRPA1 protein (Figures 4C and 4D).

(F) CNS expression pattern of dTrpA1C/D-GAL4;UAS-mCD8GFP (third instar). Expression is specific to the projections of mdIV neurons. In the body wall, expression of dTrpA1C/D-GAL4 was also seen in multidendritic bipolar (md-bp) neurons. Bipolar neuron projections from anterior segments and potential ring gland expression were also observed.

(G) Merged image of brain expression of dTrpA1C/D-GAL4;UAS-mCD8GFP with brightfield CNS.

![Figure 5](image_url)

**Figure 5. mdIV-Specific Expression of dTRPA1-C Rescues Thermal Nociception Defects of dTrpA1 Mutants**

(A) Expression of dTrpA1-C specifically in mdIV neurons in dTrpA1 mutant background (ppk-GAL4/UAS-dTrpA1-C; dTRPA1W903*/Df(3L)ED4415) restored thermal nociception behavior (3 trials, n = 82) (Wilcoxon rank-sum test with Bonferroni correction, p < 0.0001 in comparison to B and C).

(B) The control, the UAS-dTrpA1-C transgene, in the absence of a driver in the mutant background (UAS-dTRPA1-C/+; dTRPA1W903*/Df(3L)ED4415), showed the delayed responses to a 46°C stimulus that was typical of the mutant (2 trials, n = 51).

(C) The control, ppk-GAL4 driver alone, in the mutant background (ppk-GAL4/+; dTRPA1W903*/Df(3L)ED4415), showed the delayed responses to a 46°C stimulus that was typical of the mutant (3 trials, n = 83). Error bars indicate the SEM.

To test how the biophysical properties of the isoforms identified here differ from each other, we developed a heterologous expression system. We expressed each isoform, along with the genetically encoded calcium indicator GCaMP3.0 (GCaMP) (Tian et al., 2009), in the Drosophila S2R+ cell line (Yanagawa et al., 1998). We chose to use the Drosophila S2R+ cells for our experiments because the lipid content of the plasma
Figure 6. The dTRPA1-C Isoform Is Not Activated by Temperature in Heterologous Expression System but Does Respond to AITC

Heat-induced calcium transients are observed in S2R+ cells transfected with UAS-dTrpA1-A, but not in S2R+ cells transfected with UAS-dTrpA1-C. Scale bars represent 10 μm.

(A and B) Images of representative S2R+ cells transfected with dTrpA1-A and the genetically encoded calcium indicator GCaMP3.0, displaying increased GCaMP fluorescence at an elevated temperature (38°C) in comparison to room temperature (21°C).
membrane of insect cells is distinct from that of vertebrate cells and we wished to examine the channels in an environment that would closely resemble the situation in vivo. The use of GCaMP in our experiments allowed us to specifically investigate Ca\(^{2+}\) responses of transfected cells. This was important because the transfection efficiency of S2R\(^+\) cells was relatively low. Consistent with previous experiments showing that dTRPA1-A is a warmth-activated channel, we observed a dramatic temperature induced increases of GCaMP fluorescence in S2R\(^+\) cells transfected with dTRPA1-A (Figures 6A–6C, 6H, and 6I). The majority of cells expressing dTRPA1-A displayed GCaMP fluorescence increases of 50\% or greater during the heat ramp, with an average increase of 122\% (Figures 6H and 6I). In contrast, cells transfected with dTRPA1-C did not respond to temperature increases up to 42 °C with elevated GCaMP fluorescence (Figures 6D, 6E, and 6G–6I). The absence of a temperature response in cells expressing dTRPA1-C was not due to dTRPA1-C being a nonfunctional channel, as a similar proportion of cells expressing dTRPA1-A or dTRPA1-C responded to application of the dTRPA1 agonist AITC (70\% and 67\%, respectively) with a similar peak response (100\% and 125\% of baseline, respectively) (Figures 6F–6I). Since the mammalian TRPA1 is activated by cold with a threshold of 17 °C, we also tested whether the dTRPA1-C isoform would respond to noxious cold. Within the cooling ramps of 22 °C–15 °C, no Ca\(^{2+}\) responses were detected, suggesting that dTRPA1-C is not sensitive to cold (data not shown).

In order to further validate the heat insensitivity of the dTRPA1-C isoform revealed in Ca\(^{2+}\) imaging experiments, we performed whole-cell patch-clamp recordings on the Drosophila S2R\(^+\) cells expressing the channel. Cells were voltage clamped at −80 mV, and whole-cell currents were monitored every 3 sec with a 600 ms ramp from −80 to +80 mV. After stabilization of the current at 24.3 °C, the bath temperature was raised to 33 °C and then returned to 24.3 °C. Cells were then treated with the dTRPA1 agonist AITC. As shown in Figures 7A–7C, cells expressing dTRPA1-A showed dramatic current increases at elevated temperatures (33 °C) and in response to the dTRPA1 agonist AITC. In contrast, cells expressing dTRPA1-C did not respond to temperature increases to 33 °C, but did show the expected robust currents upon application of AITC. At temperatures greater than 33 °C, the seal could not be maintained, and this technical limitation prevented analysis of whole-cell currents at the higher temperature ranges. Nevertheless, the absence of temperature responses measured in Ca\(^{2+}\) imaging experiments on dTRPA1-C-expressing cells indicates that the channel does not show meaningful responses to temperature within the measured range of 15 °C–42 °C. Combined, these results demonstrate that the dTRPA1-C isoform has thermosensory properties distinct from the canonical, warmth-activated dTRPA1-A isoform and further suggest the possibility that dTRPA1-C is not a temperature-sensitive TRP at all.

The surprising lack of a heat response in dTRPA1-C-expressing cells and the existence of the three other dTRPA1 splice variants provided with us with an opportunity to investigate the contributions of the distinct alternatively spliced domains in heat responses. As with dTRPA1-C, we found that cells transfected with dTRPA1-B lacked Ca\(^{2+}\) responses to temperature in the 20 °C–42 °C temperature range (Figures 6H and 6I). Also, as with dTRPA1-C, cells expressing dTRPA1-B still showed Ca\(^{2+}\) responses to AITC, indicating that it was an active channel (Figures 6H and 6I). In contrast, S2R\(^+\) cells transfected with the dTRPA1-D isoform showed responses to both temperature and AITC (Figures 6H and 6I). Interestingly, cells expressing the dTRPA1-D isoform showed Ca\(^{2+}\) responses significantly above baseline beginning at a temperature of 34 °C (Figure 6J). This temperature is significantly higher than the known temperature threshold of the dTRPA1-A isoform (27 °C) but still lower than the thermal nociception threshold of 39 °C. Interestingly, this 34 °C Ca\(^{2+}\) response of dTRPA1-D-transfected cells, matches the thermal alldynia threshold of larvae exposed to UV-C radiation (Babcock et al., 2009), making this isoform a good candidate for mediating alldynia responses.

These experiments reveal that the C-terminal TAC that is shared between dTRPA1-A and dTRPA1-D, encoded by exon 12 of the locus, is essential for the heat responses of these isoforms (schematically represented in red on the protein structure of dTRPA1-D [Figure 4D]). Conversely, the equivalent domain of...
dTRPA1-B and dTRPA1-C interfere with heat responses. Interestingly, the C-terminal TAC of the heat-insensitive dTRPA1-B and dTRPA1-C shows higher sequence similarity with vertebrate TRPA channels (Figure S2D) relative to the heat-sensitive TAC of dTRPA1-A and dTRPA1-D. Future analyses will allow precise investigation into which of the amino acids that vary between the C-terminal TACs are critical for temperature-mediated gating of these channels.

While the molecular mechanisms of the extreme temperature sensitivity of thermoTRP channels are still largely unknown, some progress has been made. C-terminal truncations of TRPV1 change the functional properties of this channel (Vlachova et al., 2003), and swapping the C terminus of heat-sensitive TRPV1 with that of cold-sensitive TRPM8 exchanges the temperature responses of the channels. Interestingly, the C terminus is not essential for TRPV1 capsaicin responsiveness or TRPM8 menthol responsiveness (Vlachova et al., 2003).

More recently, the pore region of TRPV channels has been suggested to be critical for temperature activation. Residues in the sixth transmembrane and pore region of TRPV3 are required for its heat activation (Grandl et al., 2008). Consistent with this, mutations in the outer pore region of TRPV1 also specifically impair temperature activation (Grandl et al., 2010).

The N terminus of the rattlesnake TRPA1 channel has been found to contain elements important for heat sensitivity (Cordero-Morales et al., 2011). Artificially constructed chimeric proteins between rattlesnake TRPA1, and human TRPA1, showed that the heat-sensitive properties of the rattlesnake channel could be transferred to the heat-insensitive human channel. The heat-responsive elements of the rattlesnake channel appear to lie within the ankyrin-repeat-containing region of the protein. Importantly, two separable elements of the rattlesnake ankyrin repeat domain were found to contribute to the temperature-response properties of the artificially constructed chimeric proteins. Artificial chimeric channels made between hTRPA1 and...
and dTRPA1 suggested that amino acids 400–612 of dTRPA1-A could confer heat sensitivity to hTRPA1 (Cordero-Morales et al., 2011). This region is distinct from the 37 amino acids of dTRPA1-A and dTRPA1-D that are required for heat sensitivity in the naturally occurring dTRPA1 variants. An interesting possibility is that the C-terminal TAC domain of dTRPA1-A and dTRPA1-D interacts with ankyrin repeats in the context of the native dTRPA1 channels.

**dTRPA1-A** is activated by a temperature range of 24°C–29°C, while dTRPA1-B and dTRPA1-C did not respond to temperature changes within the range of 15°C–42°C. This indicates that critical sequences important for heat activation reside in the 37 amino acid residues that are unique to the TRPA1-A isoform. However, the N-terminal TAC of dTRPA1-A must also contribute to temperature responses, because when this segment was replaced with the N-terminal TAC of the D isoform, the threshold of the temperature response was increased to approximately 34°C.

Our results indicate that no single domain of the dTRPA1 channel can completely explain its thermal-response properties. Complex allosteric interactions between the N-terminal TACs and C-terminal TAC are likely to play a role. These interactions are likely to depend on the context of intervening ankyrin repeats. Future detailed structural analyses of the four TRPA1 variants that we describe here will allow for the unraveling of these mechanisms.

**EXPERIMENTAL PROCEDURES**

**Fly Strains and Husbandry**

The following fly strains were used: w; ppk-GAL4, w; UAS-dTrpA1-A, iso w^1118^; UAS-dTrpA1RNAi (VDRC GD collection transformant ID 37249), w; Df(3L)Ed4415/TM6b, w; dTrpA1^W903*/TM6b, w; UAS-mCD8::GFP, UAS-dicer2, and UAS-ChannelRhodopsin-2:eYFP line C (UAS-ChR2::eYPinleC), Drosophila stocks were raised on standard cornmeal molasses fly food medium at 25°C.

**Nociception Assays**

The thermal nociception behavioral tests were performed as described previously (Caldwell and Tracey, 2010; Hwang et al., 2007; Tracey et al., 2003; Zhong et al., 2010). The mechanical nociception behavioral tests were performed as described previously (Caldwell and Tracey, 2010; Hwang et al., 2007; Tracey et al., 2003; Zhong et al., 2010).

**Gentle Touch Assay**

The gentle touch behavioral tests were performed as described previously (Keman et al., 1994; Zhong et al., 2010).

**Confocal Microscopy**

For the visualization of GAL4 expression patterns via confocal microscopy, dTrpA1-C/D-GAL4, UAS-mCD8::GFP larvae were anesthetized with ether until immobilized and mounted in glycerol. Brains of third instar larvae were dissected in PBS and fixed in 4% parafomaldehyde for 30 min prior to imaging.

**S2R+ Cell Culture, Calcium Imaging, and Electrophysiology**

Drosophila S2R+ cell line was maintained in Schneider’s Drosophila medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum. Cells grown on coverslips (Warner Instruments, #1.5 glass coverslip, 25 mm round) in 6-well tissue culture plates (Falcon) were transfected with Ubiquitin-GAL4 (0.75 μg DNA per well), UAS-GCADMP3.0 (0.5 μg DNA per well), and UAS-dTrpA1-A, UAS-dTrpA1-B, UAS-dTrpA1-C, or UAS-dTrpA1-D (0.25 μg DNA per well for each isoform) with the use of Cellfectin II Reagent (Invitrogen). For electrophysiology experiments, UAS-mCD8::GFP (0.25 μg DNA per well) was used in the transfection in place of UAS-GCamp3.0. Imaging was conducted 72 hr after the transfection. Coverslips were assembled in an imaging chamber (Warner Instruments, Series 20 Chamber Platform P-2) and gently rinsed with HL3 saline (70 mM NaCl, 5 mM KCl, 1.5 mM CaCl2, 20 mM MgCl2, 10 mM NaHCO3, 5 mM trehalose, 115 mM sucrose, and 5 mM HEPES [pH 7.2]) (Stewart et al., 1994). An inline solution heater (Warner Instruments, model SH-27B) was used to flow heated or cooled HL3 through the chamber to deliver temperature ramps. A thermocouple (Warner Instruments, TA-29) was connected to a data-acquisition board (Warner Instruments, TC-324B) and placed in the imaging chamber to continuously monitor the bath temperature. For each heat-response experiment, the bath temperature was increased from room temperature (<24°C) to 38°C–42°C and then cooled back to room temperature. Microscopy was performed on a Zeiss LSM 5 Live confocal system with a 20X Plan-Apochromat lens N/A 0.8 and 488 nm laser. Images were collected at 0.2–1 Hz during the temperature ramp and AITC application. The data were analyzed with the Zeiss LSM software package. Individual cells were selected as regions of interest. Baseline fluorescence for each cell was calculated by determining the mean fluorescence of all time points prior to heat ramp or AITC application (>30 sec). Peak fluorescence was determined for each cell at temperatures elevated above 24°C (for the analyses of heat responses) or after AITC application (for analyses of responses to AITC). ΔF/F0 was calculated with the formula 100% × (F-F0)/F0 where F was the fluorescence intensity at each time point and F0 was the average baseline fluorescence intensity before the introduction of any manipulation. For each imaging experiment, n ≥ 25 transfected cells, except for the measurement of dTrpA1-A-transfected cells’ responses to AITC (n = 7) and the measurement of GCaMP-only cells’ responses to temperature (n = 6).

Whole-cell voltage clamp was performed on transfected S2R+ cells plated on coverslips. Transfected cells selected for recordings were identified by mCD8::GFP fluorescence. The intracellular solution contained (in mM) cesium methanesulfonic acid 135, CsCl 5, EGTA 0.5, MgCl2 1, Mg-ATP 4, HEPES 10; adjusted to pH 7.3 with CsOH. Extracellular solution contained (in mM) NaCl 135, KCl 5, CaCl2 2, HEPES 5, Glucose 10, adjusted to pH 7.3 with NaOH. Patch pipette resistance ranged from 4 to 5.5 MΩ. Recordings were obtained with the EPC 10 USB patch amplifier (HEKA Instruments, data), and data were collected with Patchmaster (HEKA Instruments). The liquid junction potential for these recordings was not corrected, and cells were discarded if series resistance exceeded 10 MΩ. The recordings were obtained at room temperature (24.3°C) except during heat stimulation, as indicated in Figure 7. The thermal stimulation was applied by increasing the recording chamber solution’s temperature with a preheated solution via an inline heater with the use of a Temperature Controller (TC-324B, Warner Instruments), and temperature was monitored with a thermocouple (TA-32, Warner Instruments) placed in a recording chamber near the recorded cells. Data acquisition of thermistor outputs (100 mV/°C) were collected simultaneously with heat-activated current signals. Cells were held at −80 mV, and currents were monitored every 3 sec in response to a linear ramp from −80 mV to +80 mV over 600 ms. A brief 5 mV hyperpolarizing step was performed at the end of each sweep to monitor membrane resistance and assess the stability of the access resistance throughout the experiment. For statistical analysis, the current amplitude was normalized to each cell capacitance.

**Molecular Cloning**

For the generation of dTrpA1-C/D-GAL4, PCR was performed from a BAC clone template with the forward primer 5′-CACCCCATCCTTCGAGCTGGAG-3′ and the reverse primer 5′- GACCGCTGTAGACTCCGTTG-3′. The resulting PCR product was then cloned into pENTR/D-TOPO (Invitrogen) and then into the Drosophila pcSaper-DES6 Gateway destination vector with the use of Clonase II enzyme (Invitrogen). This construct was used for the generation of transgenic animals by the transposase-mediated transformation of w^1118^ flies. Expression patterns of two independent transformants were analyzed by crossing to UAS-mCD8GFP and showed similar patterns.

For the cloning of dTrpA1-C, RT-PCR was performed from total RNA extracted from a mixed population of first and second instar Canton S larvae.
Oligo (dT)12-18 (Invitrogen) and primer 5'-CTACATGCTTATGGAAGCTCAG
GGCG-3' mix were used as primers for reverse transcription, and first-strand
cDNA synthesis used SuperScriptTM II RT (Invitrogen). For amplification of
cDNA, PCR was performed with the use of the forward primer 5'-ATGCCCA
AGCTCTACAACGGAAGCTCTA-3' and the reverse primer 5'-CTACATGCCTTTA
TTGAAGCTCAGGGCG-3'. The PCR product was then reamplified with the same pair of primers. The dTrpA1-C PCR product was cloned into TOPO-XL
(Invitrogen) and fully sequenced. TOPO-XL-dTrpA1-C was further subcloned into pUAST construct with the use of the EcoRI restriction site. For the cloning of dTrpA1-D, the forward primer used was 5'-CACCATGCCAAGCTCTTAACA
ACGGAG-3' and the reverse primer used was 5'-CTACATGCCTTTAATGGAAGCTCAG
GGCG-3'. The PCR parameters were as follows: 98°C for 30 sec, 98°C for 7 sec, 68°C for 30 sec, and 72°C for 2 min; steps 2-4 repeated 29 times (for a total of 30 cycles), followed by 72°C for 8 min.

For construction of the pUAST-dTrpA1-B plasmid, a Nhel/XbaI fragment containing the 3' end of the dTrpA1-C cDNA and sequence encoding the C-terminal TAC region was excised from the pUAST-dTrpA1-C plasmid and ligated with T4 DNA ligase (New England Biolabs) into the a pUAST-dTrpA1-A plasmid cut with Nhel/XbaI. For construction of the pUAST-dTrpA1-D plasmid, a Nhel/XbaI fragment containing the 3' end of the dTrpA1-A cDNA and sequence encoding the C-terminal TAC region was excised from the pUAST-dTrpA1-A plasmid and ligated with T4 DNA ligase (New England Biolabs) into the a pUAST-dTrpA1-C plasmid cut with Nhel/XbaI.

Transgenic Flies
The pcAsper-DEST6-dTrpA1-C/D-GAL4 construct was injected by the Duke University Model System Genomics core facility for P-element-mediated transfor-
mation. This particular line used in this research is an insertion on the second chromosome. Injections for dTrpA1BAC (CH922-154N09) were performed by GenetiVision via PhiC31-mediated chromosome integration with VK37(2L)
22A3 as the docking site (Venken et al., 2006). The pUAST-dTrpA1-C construct was injected by GenetiVision for P-element-mediated transformation.

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