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Abstract

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Thermosensory and Nonthermosensory Isoforms of *Drosophila melanogaster* TRPA1 Reveal Heat-Sensor Domains of a ThermoTRP Channel

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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 A δ 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). The TRPA1 channel is activated by many irritant chemicals, such as cinnamaldehyde, allyl isothiocyanate (AITC) (mustard oil), allicin (garlic), and acrolein (tear gas) (Bautista et al., 2006; Bautista et al., 2005). Many of these chemicals are reactive electrophiles that are thought to activate TRPA1 through covalent modification of cysteines (Bautista et al., 2006; Macpherson et al., 2007). Indeed, TRPA1 knockout mice show profound defects in avoiding these normally noxious compounds (Bautista et al., 2006; Kwan et al., 2006).

Two recent studies have now shown that *Drosophila TrpA1* (dTrpA1) is required for thermal 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,

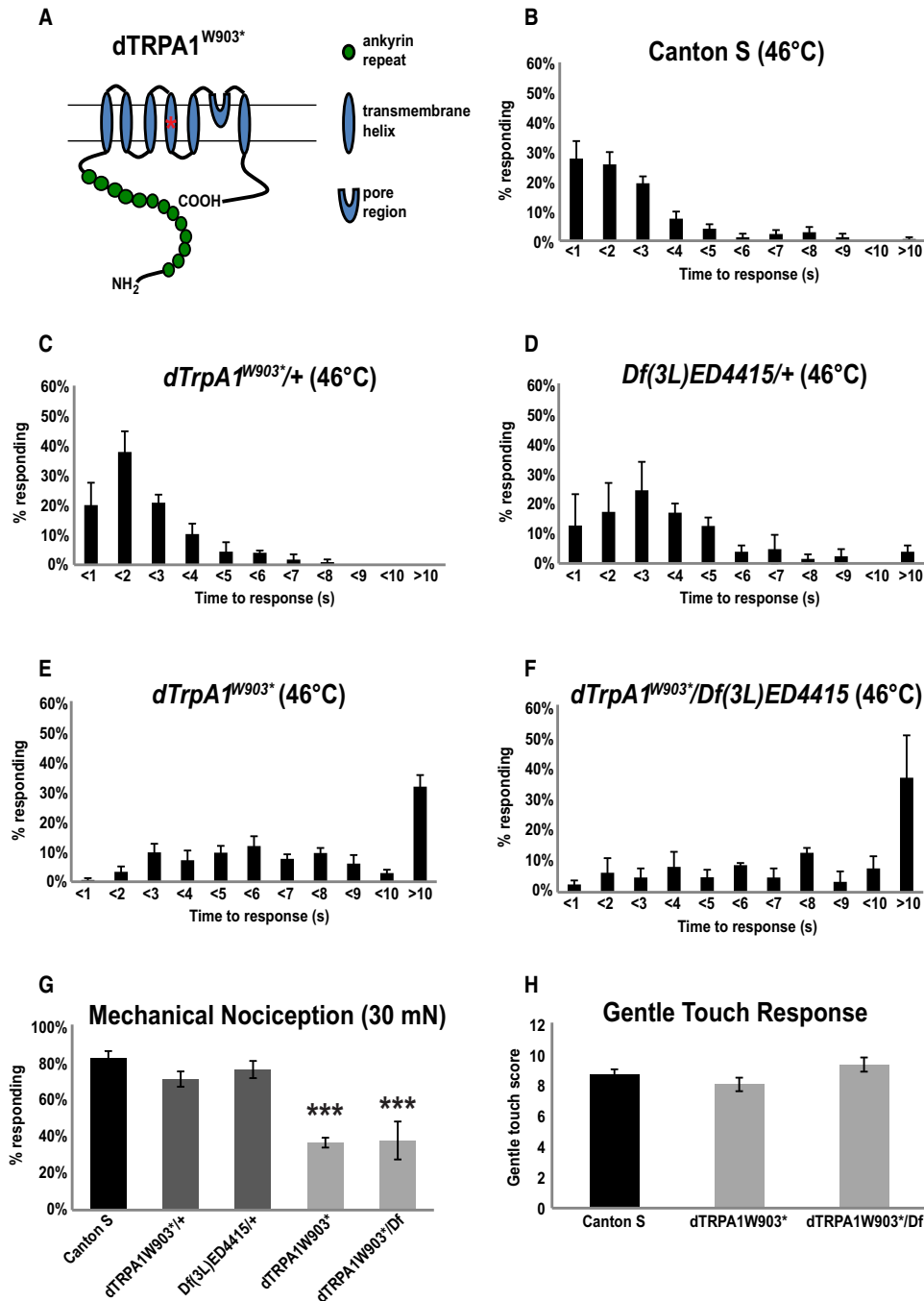


Figure 1. dTrpA1 Is Required for Thermal and Mechanical Nociception

(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 dTrpA1^{W903*}.

(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 dTrpA1^{W903*/+} 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) dTrpA1^{W903*} mutant larvae (4 trials, n = 149) showed severely delayed nociception responses in comparison to control strains Canton S and dTrpA1^{W903*/+} (Wilcoxon rank-sum test with Bonferroni correction, p < 0.0001). (F) dTrpA1^{W903*/Df(3L)ED4415} larvae (3 trials, n = 67) showed delayed NEL in comparison to control strains Canton S, dTrpA1^{W903*/+} and Df(3L)ED4415/+, indicating that Df(3L)ED4415 failed to complement the NEL defects of dTrpA1^{W903*} (Wilcoxon rank-sum test with Bonferroni correction, p < 0.0001).

(G) dTrpA1 is required for mechanical nociception. A significantly reduced proportion of dTrpA1^{W903*} [3 trials, n = 97, Pearson's chi-square test for independence with Bonferroni correction, p < 0.001 (***) in comparison to Canton S or dTrpA1^{W903*/+}] and dTrpA1^{W903*/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 ankryin 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 methanesulfonate (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 *dTrpA1^{W903*}*, 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 *dTrpA1^{W903*}* mutation from other unlinked EMS-induced mutations that might be present in the mutagenized strain, we outcrossed the *dTrpA1^{W903*}* mutant chromosome to the *Df(3L)ED4415* strain for six generations.

dTrpA1 Mutants Are Defective for Both Thermal and Mechanical Nociception

We found that the outcrossed *dTrpA1^{W903*}* mutant animals showed pronounced defects in behavioral assays for nociception. *Drosophila* larvae produce stereotyped nocifensive escape locomotion (NEL) behavior in response to noxious thermal or mechanical stimuli (Hwang et al., 2007; Wheeler et al., 2002; Zhong et al., 2010). Wild-type animals gently touched with a 46°C probe initiate NEL behavior within 3 sec (Figure 1B) (Babcock et al., 2009; Hwang et al., 2007; Tracey et al., 2003). In contrast, we observed that *dTrpA1^{W903*}* mutant larvae displayed

a significantly delayed response. Many of the mutants failed to initiate the escape behavior within 10 sec (Figure 1E). The heterozygous *dTrpA1^{W903*/+}* larvae showed normal responses to noxious heat, and *Df(3L)ED4415/+* larvae had only mild defects (Figures 1C and 1D), indicating a recessive mutant phenotype (Figure 1C). The mutant phenotype mapped to the *dTrpA1* locus, as *Df(3L)ED4415* failed to complement *dTrpA1^{W903*}* (Figure 1F).

We also tested the *dTrpA1* mutants for mechanical nociception responses (Figure 1G). Compared to wild-type and heterozygous controls, which produced robust nocifensive responses to stimulation with a 30 mN von Frey fiber, both the *dTrpA1^{W903*}* and *dTrpA1^{W903*/Df(3L)ED4415}* larvae were significantly less responsive (Figure 1G). This decreased response was specific to mechanical nociception, as *dTrpA1* mutant larvae showed normal responses to gentle touch (Figure 1H). In addition, gross motor functions of *dTrpA1* mutants appeared normal (data not shown) (Rosenzweig et al., 2005).

A 21 kb piece of genomic DNA that included the entire *dTrpA1* locus (and the neighboring gene, *mcm7*) completely rescued the *dTrpA1* thermal and mechanical nociception defects (Figures 2A and 2E). This further narrowed the genetic aberration causing the nociception defects of the mutant down to one of two genes, *dTrpA1* and *mcm7*.

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 to AITC 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 genetic 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 *dTrpA1^{W903*/+}* larvae showed NEL responses to 30 mN Von Frey fibers relative to wild-type Canton S larvae (5 trials, $n = 120$), *dTrpA1^{W903*/+}* 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 *dTRPA1^{W903*}* ($n = 16$) and *dTRPA1^{W903*/Df(3L)ED4415}* ($n = 21$) were not significantly different (single-factor ANOVA, $p = 0.13$).

In (B–H), error bars indicate the SEM.

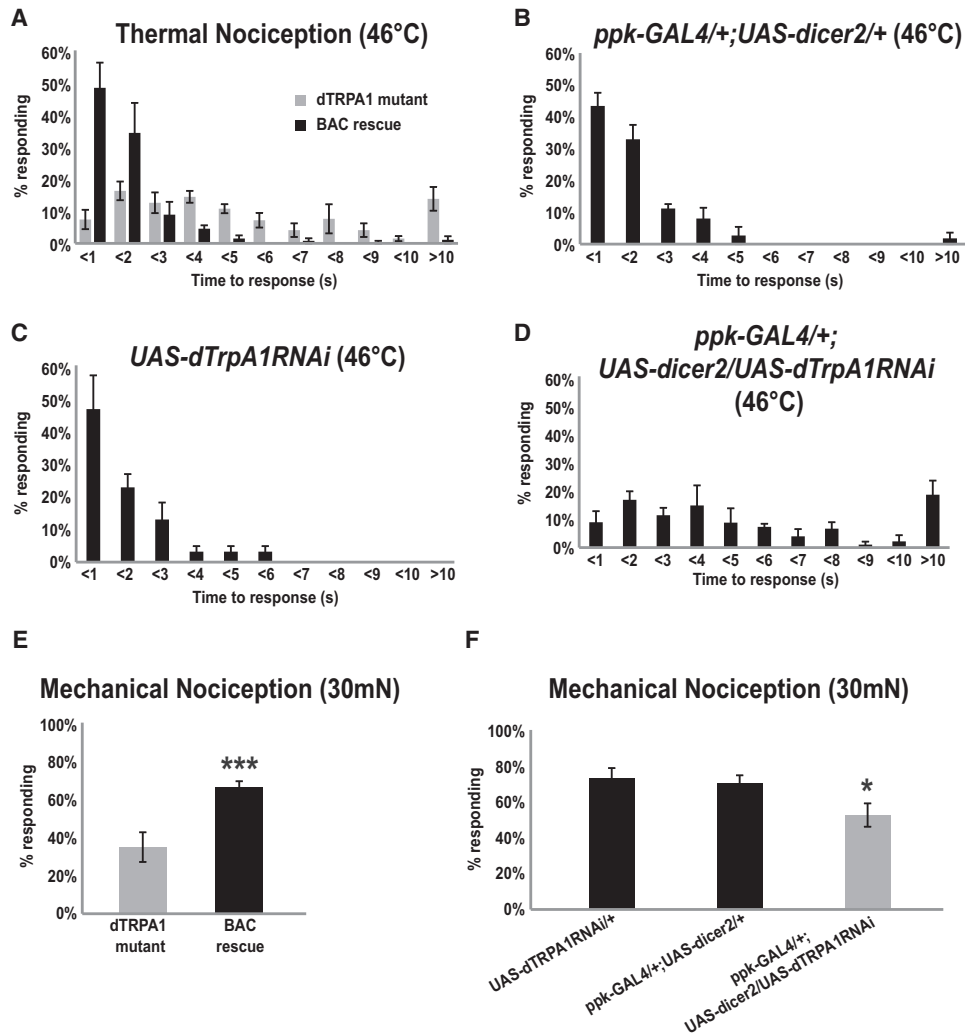


Figure 2. Genomic Rescue of *dTrpA1* and Nociceptor-Specific RNAi Knockdown of *dTrpA1*

(A) In the thermal nociception assay, the *dTrpA1-BAC/+; dTrpA1^{W903*/Df(3L)ED4415}* larvae (3 trials, n = 143) showed rescued nocifensive behavior resembling the wild-type, while *dTrpA1^{W903*/Df(3L)ED4415}* mutants tested side by side still showed delayed responses (3 trials, n = 180) (Wilcoxon rank-sum test, p < 0.01).

(B–D) The nociception responses of larvae with *dTrpA1RNAi* knockdown in mdIV neurons (*ppk-GAL4/+; UAS-dicer2/UAS-dTrpA1RNAi*, 3 trials, n = 112) are significantly delayed in comparison to the control groups (*ppk-GAL4/+; UAS-dicer2/+*, 3 trials, n = 113; *UAS-dTrpA1RNAi/+*, 3 trials, n = 49) (Wilcoxon rank-sum test with Bonferroni correction, p < 0.0001).

(E) In the mechanical nociception assay, *dTrpA1-BAC/+; dTrpA1^{W903*/Df(3L)ED4415}* larvae (3 trials, n = 139) showed rescued nociception responses upon stimulation with a 30 mN mechanical force relative to *dTrpA1^{W903*/Df(3L)ED4415}* mutants [3 trials, n = 111, Pearson's chi-square test for independence with Bonferroni correction, p < 0.001(***)].

(F) Nociceptor-specific knockdown in *dTrpA1RNAi* larvae (*ppk-GAL4/+; UAS-dicer2/UAS-dTrpA1RNAi*, n = 124) showed reduced mechanical nociception responses to a 30 mN mechanical force in comparison to control strains that had driver alone (*ppk-GAL4/+; UAS-dicer2/+*, n = 110) or to *UAS-dTrpA1* without the driver (*UAS-dTrpA1RNAi/+*, n = 84) [Pearson's chi-square test for independence with Bonferroni correction, p < 0.01(**) in comparison to *UAS-dTrpA1RNAi/+*, p < 0.05(*) in comparison to *ppk-GAL4/+; UAS-dicer2/+*].

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-dTrpA1-A/+*) 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

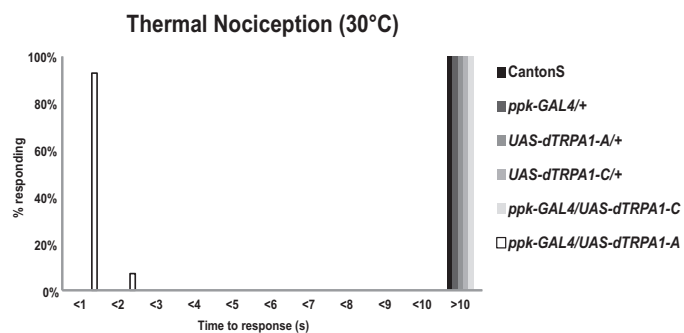


Figure 3. Expression of dTRPA1-A, but not dTRPA1-C, in mdIV Neurons Lowers the Thermal Nociception Threshold

Wild-type Canton S larvae ($n = 86$), *UAS-dTrpA1-A/+* larvae ($n = 54$), *ppk-GAL4/+* larvae ($n = 37$), *UAS-dTRPA1-C/+* larvae ($n = 30$), and *ppk-GAL4/UAS-dTRPA1-C* larvae ($n = 30$) did not show nocifensive responses to 30°C stimuli within 10 sec. However, *ppk-GAL4/UAS-dTrpA1-A* larvae ($n = 28$) showed robust nocifensive responses to 30°C stimuli, with most of the larvae responding within 1 sec (Wilcoxon rank-sum test with Bonferroni correction, $p < 0.0001$ in comparison to all other groups).

produced NEL in response to 30°C heat (Figure 3). The behavior of larvae expressing *dTRPA1-A* in nociceptors causes thermal allodynia 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 knock-down 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-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

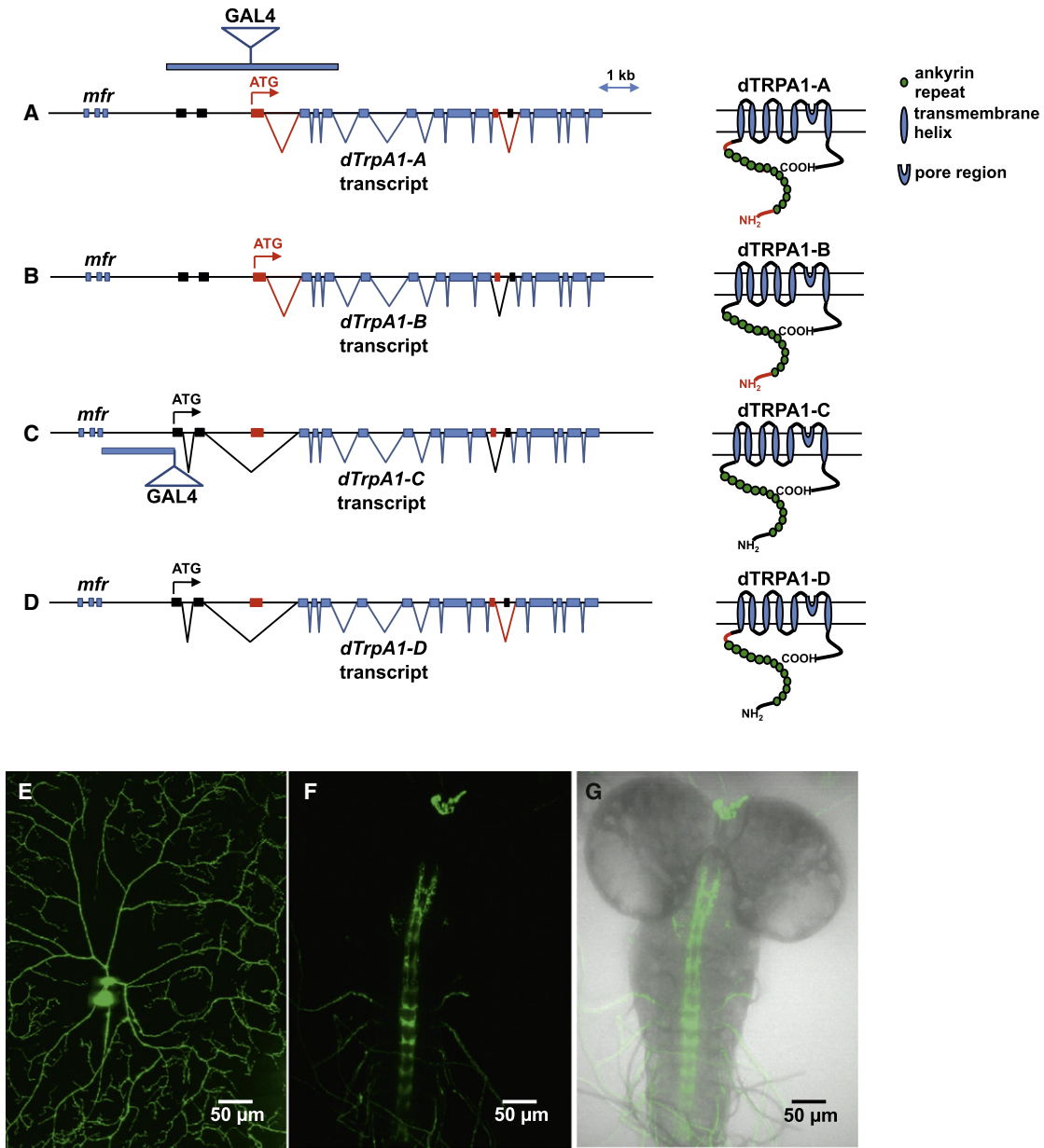


Figure 4. Gene and Protein Structures of Four Isoforms of *dTrpA1* and the *dTrpA1-C/D* Expression Pattern

(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 *dTrpA1^{W903*}/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 for *Drosophila* thermal 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 *dTrpA1^{W903*}* 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-undefined 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).

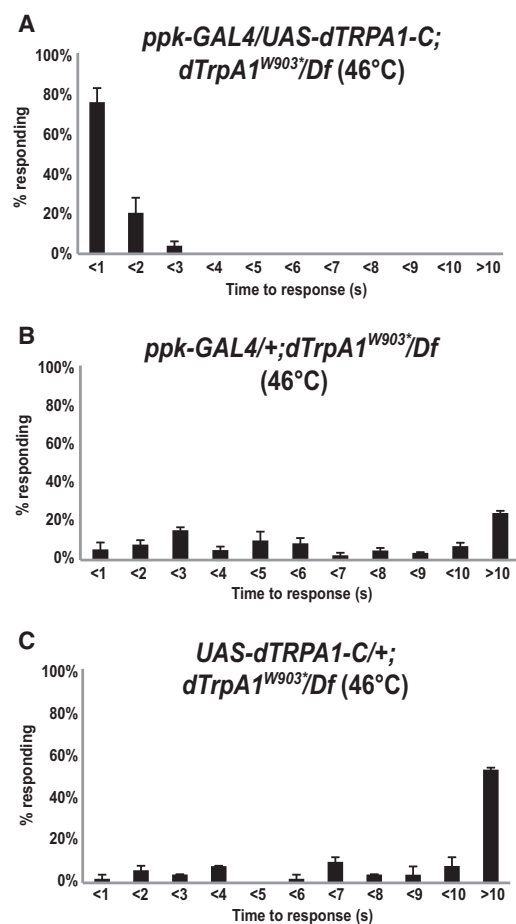


Figure 5. mdIV-Specific Expression of dTRPA1-C Rescues Thermal Nociception Defects of *dTrpA1* Mutants

(A) Expression of *dTrpA1-C* specifically in mdIV neurons in *dTrpA1* mutant background (*ppk-GAL4/UAS-dTrpA1-C; dTrpA1^{W903*}/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/+; dTrpA1^{W903*}/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/+; dTrpA1^{W903*}/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

(F) CNS expression pattern of *dTrpA1-C/D-GAL4;UAS-mCD8GFP* (third instar). Expression is specific to the projections of mdIV neurons. In the body wall, expression of *dTRPA1-C/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 *dTrpA1-C/D-GAL4;UAS-mCD8GFP* with brightfield CNS.

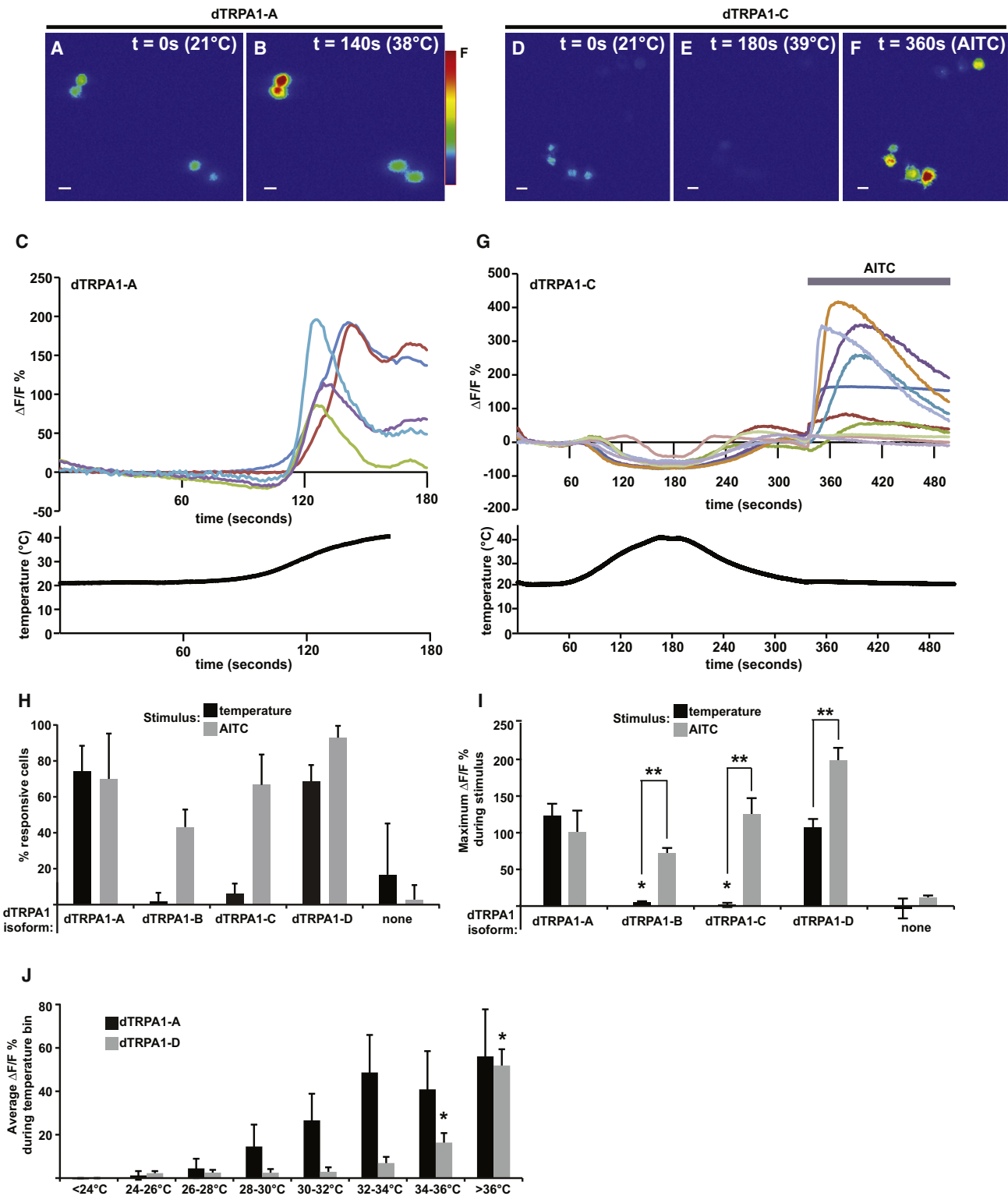


Figure 6. The dTRPA1-C Isoform Is Not Activated by Temperature in Heterologous Expression System but Does Respond to AITC

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 μ m.

(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 allodynia threshold of larvae exposed to UV-C radiation (Babcock et al., 2009), making this isoform a good candidate for mediating allodynia 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

(C) Representative traces from *dTrpA1-A*-transfected S2R+ cells showing GCaMP fluorescence (above) in response to a temperature ramp (below). Each colored trace represents an individual cell.

(D–F) Images of representative S2R+ cells transfected with *UAS-dTrpA1-C* and the genetically encoded calcium indicator *UAS-GCaMP3.0*, displaying no increase GCaMP fluorescence at an elevated temperature (39°C) in comparison to room temperature (21°C), but a large increase in fluorescence in response to allyl isothiocyanate (AITC).

(G) Representative traces from *dTrpA1-A*-transfected S2R+ cells showing GCaMP fluorescence (above) in response to a temperature ramp (below) and application of 0.03 mM AITC (gray bar). Each colored trace represents an individual cell.

(H) Percentage of S2R+ cells transfected with *dTrpA1-A* and GCaMP, *dTrpA1-B* and GCaMP, *dTRPA1-C* and GCaMP, *dTrpA1-D* and GCaMP, or GCaMP alone that displayed a peak GCaMP $\Delta F/F_0\%$ of greater than 50% in response to increased temperature (black bars) and 0.03 mM AITC (gray bars). Cells expressing dTRPA1-A or dTRPA1-D responded to increased temperature and AITC. Cells expressing dTRPA1-B or dTRPA1-C responded to AITC, but few responded to increased temperature. Few GCaMP-only cells responded to increased temperature or AITC. Errors indicate 95% confidence intervals (as determined by Wilson's estimate).

(I) Average peak $\Delta F/F_0\%$ of S2R+ cells transfected with *dTrpA1-A* and GCaMP, *dTrpA1-B* and GCaMP, *dTRPA1-C* and GCaMP, *dTrpA1-D* and GCaMP, or GCaMP alone in response to increased temperature (black bars) or 0.03 mM AITC (gray bars). Cells expressing dTRPA1-A showed an average peak $\Delta F/F_0\%$ that was significantly greater than that of cells expressing dTRPA1-C or dTRPA1-B (as determined by Student's t test; single asterisks indicate $p \leq 0.001$). Cells expressing dTRPA1-B, dTRPA1-C, and dTRPA1-D each showed an average peak $\Delta F/F_0\%$ in response to AITC that was significantly greater than the peak in response to heat (as determined by Student's t test; double asterisks indicate $p \leq 0.005$). Error bars indicate SE.

(J) Average $\Delta F/F_0\%$ of all data points within given temperature bins for cells transfected with *dTrpA1-A* and GCaMP (black bars) or *dTrpA1-D* and GCaMP (gray bars). Cells expressing dTRPA1-D showed significantly increased GCaMP fluorescence at the 34°C–36°C and >36°C temperature range when compared to the 24°C–26°C range (as determined by one-way ANOVA with Dunnett's posttest; asterisk indicates $p \leq 0.05$). Error bars indicate SE.

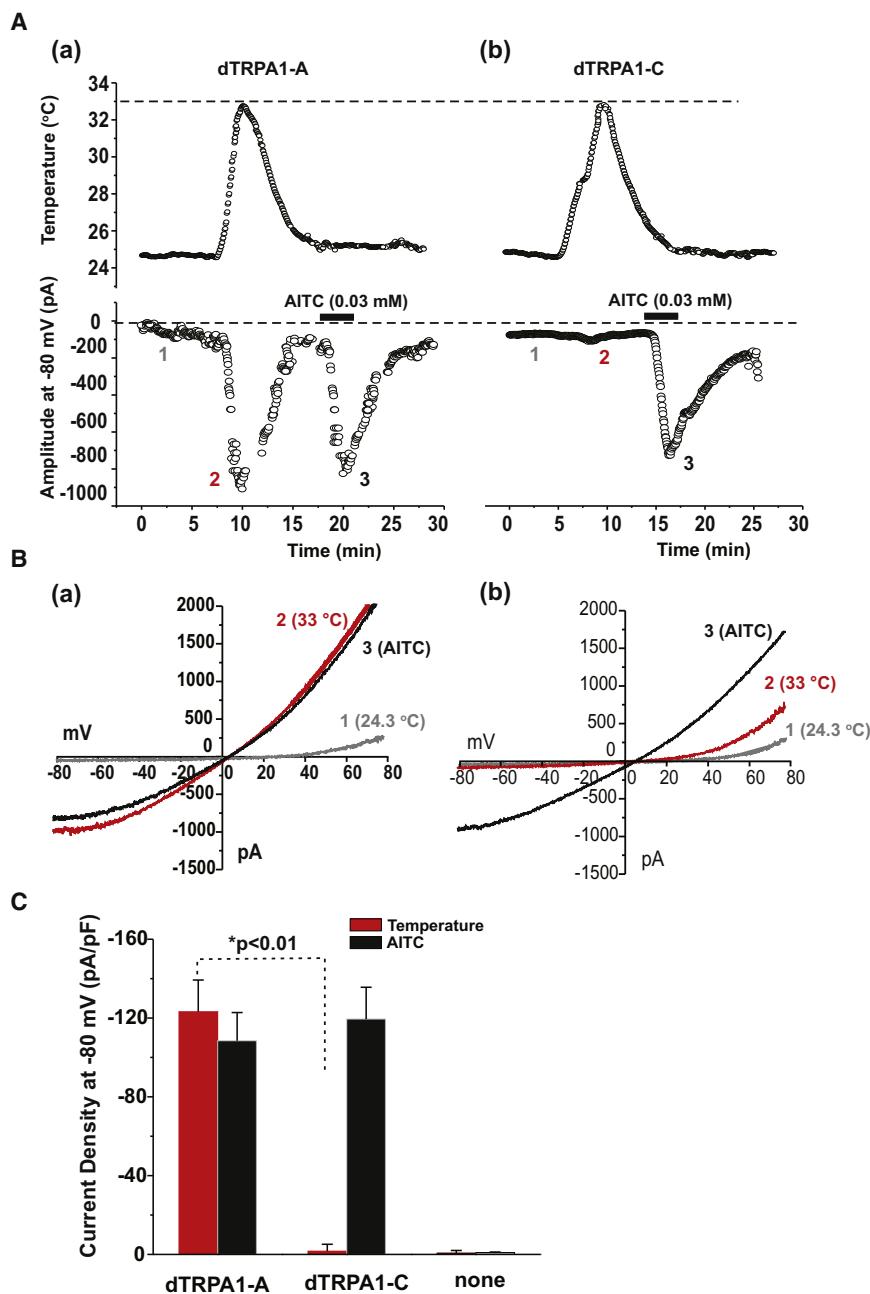


Figure 7. Patch-Clamp Recordings on *Drosophila* S2R+ Show that the dTRPA1-C Isoform Is Not Activated by Temperature but Does Respond to AITC

(A) Diary plot of current amplitude at -80 mV from an S2R+ cell transfected with *dTrpA1-A* (a) or *dTrpA1-C* (b) during a temperature challenge and after application of 0.03 mM AITC. Measured temperature of the bath solution is shown in the top panels. Note the absence of a response to temperature increase for *dTrpA1-C*.

(B) Example current traces from an S2R+ cell transfected with *dTrpA1-A* (a) or *dTrpA1-C* (b) in response to a ramp protocol from -80 to $+80$ mV at 24.3°C , at 33°C , and after application of AITC at 24.3°C , taken at the indicated times (see 1, 2, and 3) in (A).

(C) Summary data showing that *dTrpA1-A*, but not *dTrpA1-C*, responded to temperature. Both *dTrpA1-A* and *dTrpA1-C* responded to AITC. For current amplitude, $n = 15$ for *dTrpA1-A*, $n = 12$ for *dTrpA1-C*; $*p < 10^{-6}$. For AITC application, $n = 9$ for *dTrpA1-A*, $n = 12$ for *dTrpA1-C*; $*p > 0.05$. For mCD8::GFP only, $n = 6$ for temperature, $n = 6$ for AITC application. Scale bars represent SE.

TRPV1 change the functional properties of this channel (Vlachová 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 (Vlachová 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

dTRPA1-B and dTRPA1-C interferes 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

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 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 w¹¹¹⁸; UAS-dTrpA1RNAi* (VDRG 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::eYFP_{lineC})*. *Drosophila* stocks were raised on standard commeal 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 (Kernan 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% paraformaldehyde 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-GCaMP3.0* (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 physiology 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). $\Delta F/F_0\%$ was calculated with the formula $100\% \times (F - F_0)/F_0$ where F was the fluorescence intensity at each time point and F_0 was the average baseline fluorescence intensity before the introduction of any manipulation. For each imaging experiment, $n \geq 25$ 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), 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-30, 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'-CACCCATTCACATTGAGTGAG-GACTAC-3' and the reverse primer 5'-GACCGTGTAGACTCCGTTG-3'. The resulting PCR product was then cloned into pENTR/D-TOPO (Invitrogen) and then into the *Drosophila* pCaSpeR-DEST6 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 *w¹¹¹⁸* 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'-CTACATGCTCTTATTGAAGCTCAGGGCG-3' mix were used as primers for reverse transcription, and first-strand cDNA synthesis used SuperScript™ II RT (Invitrogen). For amplification of cDNA, PCR was performed with the use of the forward primer 5'-ATGCCCAAGCTCTACAACGGAGTCTA-3' and the reverse primer 5'-CTACATGCTCTTATTGAAGCTCAGGGCG-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'-CACCATGCCCAAGCTCTACAACGGAG-3' and the reverse primer used was 5'-CTACATGCTCTTATTGAAGCTCAGGG-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 NheI/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 *pUAST-dTrpA1-A* plasmid cut with NheI/XbaI. For construction of the *pUAST-dTrpA1-D* plasmid, a NheI/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 *pUAST-dTrpA1-C* plasmid cut with NheI/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 transformation. This particular line used in this research is an insertion on the second chromosome. Injections for *dTrpA1BAC* (CH322-154N09) 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.

ACCESSION NUMBERS

The GenBank accession numbers for *dTrpA1-C* and *dTrpA1-D* are JN400354 and JN814911, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and can be found with this article online at doi:10.1016/j.celrep.2011.11.002.

LICENSING INFORMATION

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