Involvement of TRPV1 and TDAG8 in Pruriception Associated with Noxious Acidosis

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Itch and pain are closely related but are distinct sensations. Intradermal injection of acid generates pain in both rodents and humans; however, few studies have addressed the intriguing question of whether acid (protons) can evoke itch like other algogens by spatial contrast activation of single nociceptors. Here, we report that (i) citric acid (0.2 mol/L) pH-dependently induced a scratching response in mice when applied intradermally to nape or cheek skin, (ii) acidified buffer elevated intracellular calcium levels in dorsal root ganglion pruriceptors, and (iii) injection of intradermal citric acid (pH 3.0) into the nape induced a pruritogen-like but not algogen-like c-Fos immunoreactivity pattern in the cervical spinal cord. Using pharmacological and genetic approaches, we identified potential acid-sensing channels/receptors involved in acidic citrate-evoked itch. Results indicate that TRPV1, but neither ASIC3 nor TRPA1, is involved in the acidic citrate-induced scratching response. Furthermore, one of the proton-sensing G-protein–coupled receptors, TDAG8, was highly (~71%) expressed in Nppb^{+} dorsal root ganglion pruriceptors. Itch induced by acidic citrate, but not α-methyl-5-hydroxytryptamine, chloroquine, compound 48/80, or bile acid, was markedly decreased in TDAG8$^{-/-}$ mice. In a heterologous expression system, TDAG8 potentiated the acid-induced calcium response by regulating TRPV1. Thus, protons could evoke pruriception by acting on TDAG8 to regulate TRPV1 activation with its mechanism of future therapeutic relevance.

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INTRODUCTION

Itch is defined as an unpleasant sensation that elicits the desire or reflex to scratch. The urge to scratch the affected skin physically is the key episode defining itch and thus protects mammals against possible contact with potential "danger molecules" like parasites or irritants (Ikoma et al., 2006). *Pain* is defined as an "unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" by the International Association for the Study of Pain (1994). Although both somatosensory sensations activating sensory nerves, itch and pain can be differentiated by psychophysiological and molecular characteristics (LaMotte et al., 2014). Dorsal root ganglion (DRG) pruriceptors anatomically represent a subpopulation of DRG nociceptors positive for transient receptor potential cation channel V1 (TRPV1) (Han et al., 2013).

Recent itch studies have shown that pruritogens, such as histamine (H), serotonin (5-hydroxytryptamine, 5-HT), endothelin (ET)-1, and chloroquine, induce itch sensations via direct action on their specific receptors, H_1 receptor, $5\text{-}HT_2$ receptor, ET_A receptor, and Mas-related G-protein–coupled receptor (Mrgpr) A_3 receptor, respectively, in the DRG pruriceptors (Bautista et al., 2014). Most, if not all, DRG pruriceptors are TRPV1 expressing, and within this population, only a subset of Mrgpr^{+} pruriceptors express TRPA1. However, itch and pain are distinct because of the recent discoveries of itch-specific effectors, modulators, and neurotransmitters (Goswami et al., 2014; Liu et al., 2009; Sun et al., 2007). The labeled line theory of itch has gained strong support, because genetic knockout or pharmacological ablation of these itch-specific components impede the itch response and leave the pain behavior intact. One unsolved question is why some well-known algogens (e.g., capsaicin) also induce itch (Sikand et al., 2009). The opponent theory emphasizes that mechanical/painful scratching inhibits itching, and morphine-like analgesics suppress pain but enhance itching. The opponent action between itch and

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Abbreviations: ASIC, acid-sensing ion channel; CP48/80, compound 40/80; DRG, dorsal root ganglion; ET, endothelin; GPCR, G-protein–coupled receptor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HT, hydroxytryptamine; IB4, isolectin B4; IRES, internal ribosome entry site; MES, 2-(N-morpholino)ethanesulfonic acid; Mrgpr, Mas-related G protein-coupled receptor; $Na_V1.8$, voltage-gated sodium channel 1.8; PBS, phosphate-buffered saline

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pain could occur at the spinal cord but not DRG level (Liu et al., 2011). Pattern theory, which claims that the ultimate perceived sensation is encoded across the pattern of peripheral nerve activation before being decoded by the central brain, might provide the answer, but supporting evidence is limited (Ikoma et al., 2006). Spatial contrast theory reconciles contradictory findings of itch studies, which states that itch arises from a sharp contrast between individual nociceptors firing among the surrounding silent neighbors; pain would be felt during a more homogenous activation of surrounding nociceptors (Namer & Reeh, 2013).

Increasing evidence indicates that TRPV1 and TRPA1 are downstream effectors in histamine- and Mrgpr-dependent itching, respectively (Shim et al., 2007; Wilson et al., 2011). Histamine-induced itch is specifically reduced in *TrpV1*$^{-/-}$ but not *TrpA1*$^{-/-}$ mice, but chloroquine-induced itch is specifically impeded in *TrpA1*$^{-/-}$ but not *TrpV1*$^{-/-}$ mice. Histamine, when acting on histamine H_1 receptor, may activate phospholipase-Cβ and thus functionally couple to TRPV1 to exert itch. However, chloroquine activates Mrgpr-A3 to release G-protein βγ subunits to activate TRPA1.

Acid (protons) is a potent algogen that induces pain in humans and rodents by activating TRPV1, acid-sensing ion channels (ASICs), and proton-sensing G-protein–coupled receptors (GPCRs) such as OGR1, TDAG8, G2A, and GPR4 (Sun and Chen, 2016). Previously, we have shown that acidic citric buffer (pH 3.0) induced itch in histamine-sensitized skin in healthy people and lesional skin in patients with atopic dermatitis (Ikoma et al., 2004). However, the molecular mechanism underlying the citrate-induced itch is unknown. In this study, we provide evidence that acidic citrate can act on TDAG8 coupled with TRPV1 to induce nociception, resulting in itch-like behavior in mice.

RESULTS

Intradermal acid solution induced scratching response in mice

To verify whether protons can induce an itch-like sensation in mice, we first screened the scratching behavior in wild-type male mice by intradermal injection of formic acid. When 10 μl of formic acidic solution (pH 1.9, 3.0, or 4.7) was injected into the nape skin of mice, only at the most acidic pH of 1.9 did mice displayed scratching behavior (Figure 1a).

Because protons without a buffer system is easily diluted in the skin tissue, we next used buffered citric acid to test the scratching response. The amount of 0.2 mol/L of citric acid was adjusted with 0.2 mol/L tri-sodium citrate to a pH of 7.4, 4.7, 3.0, and 1.9. Compared with normal saline, pH-7.4 citric acid induced some scratching responses because of a reduction of free calcium, and a similar effect was also observed in mice treated with EGTA-saline (see Supplementary Figure S1 online). With H^+ concentration increased, citric acid pH-dependently increased scratching behavior in the mouse nape skin model (Figure 1b).

We compared the itch-inducing capacity of citric acid (pH 3.0) with several well-known pruritogens, including deoxycholic acid (DCA), α-methyl-5-HT, chloroquine, and compound 48/80 (CP40/80), in different groups of wild-type mice. The order of itch-producing potency in nape skin model was CP48/80 (100 μg) > chloroquine (200 μg) > α-methyl-5-HT (30 μg) > citric acid (pH 3.0) > deoxycholic acid (25 μg) (Figure 1c). Because subcutaneous acid treatment has been reported to produce pain in both humans and rodents, we verified whether citric acid (pH 3.0) was a pure pruritogen or had combined algogen properties in the mouse cheek skin assay. Compared with the pain-inducing algogen capsaicin, which induced mainly a wiping response, the itch-inducing pruritogen chloroquine induced mainly a scratching response. Citric acid induced an equivalent wiping response to capsaicin (which indicates that protons are an algogen) and significantly more scratching response than capsaicin (which indicates that protons are also a pruritogen) (Figure 1d). Chlorpheniramine, an anti-histamine drug, inhibited the citric acid-induced scratching in both nape and cheek skin models (see Supplementary Figure S2 online). Similar to humans, citric (or even formic) acid (pH 3.0)-induced scratching was enhanced in histamine-sensitized skin (Figure 1e and f). Our results support that protons not only induce nociception but also pruriception.

Acid induced calcium transients in DRG pruriceptors and generated itch-specific c-Fos immunoreactivity in the spinal dorsal horn

To harvest DRG pruriceptors for calcium imaging study, we used the double transgenic mice bearing $Na_V1.8$-Cre and the Cre reporter CAG-STOP^{floxed}-Td-tomato to label $Na_V1.8^+$ DRG neurons, which cover most populations of nociceptors and pruriceptors (Shields et al., 2012). After loading with Fura-2, Td-tomato$^+$ small- to medium-sized $Na_V1.8^+$ DRG neurons were sequentially challenged with histamine (100 μmol/L), chloroquine (1 μmol/L), acidic 1 × Hanks balanced salt solution (pH 3.0), capsaicin (10 μmol/L) and potassium chloride (75 mmol/L) (Figure 2a–c). In three independent trials, 66% (144/215) and 54% (118/215) of the selected neurons responded to capsaicin and acid, respectively. About 20% (43/215), 16% (36/215), and 14% (32/215) of the selected neurons responded to histamine, chloroquine, and both, respectively. All acid-responsive neurons were capsaicin responsive, and all histamine- and/or chloroquine-responsive neurons were acid responsive (Figure 2d). DRG pruriceptors conveyed itch signals to the secondary relay neurons, which are Npra$^+$ and Grp$^+$, in laminae I and II of the spinal dorsal horn, where c-Fos expression is induced after injection of 5-HT (Akiyama et al., 2009) or chloroquine (Zhang et al., 2014) in the nape skin model. We verified whether citric acid at pH 3.0 could generate the same itch-specific c-Fos expression. For comparison, we used capsaicin and CP48/80 as negative and positive controls, respectively, because capsaicin did not generate a scratching response and CP48/80 was the most potent pruritogen we found (Figure 1c). After 30 minutes of scratching with CP48/80 and acid treatment, condensed c-Fos immunoreactivity was observed in the lateral superficial dorsal horn, which was not observed with capsaicin and saline treatment (Figure 2e–h).

Acidic citrate-induced scratching is TRPV1 dependent

In general, itch can be classified into two categories: histamine- and non-histamine–dependent itch, modulated by downstream activation of TRPV1 and TRPA1 channels, respectively (Bautista et al., 2014). Furthermore, TRPV1 and ASIC3 in the peripheral terminals of primary sensory neurons

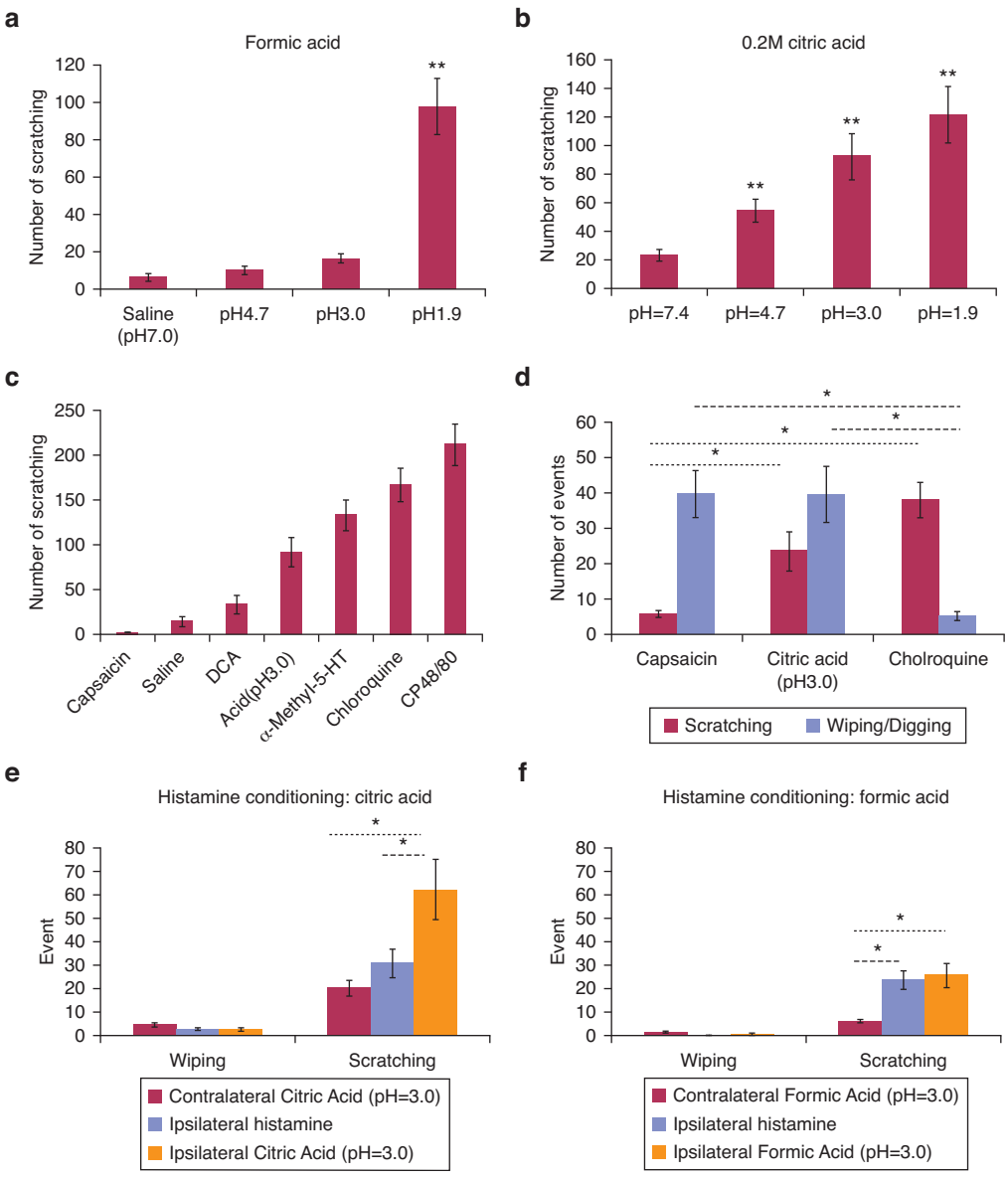

Figure 1. Proton induced scratching response in wild-type mice. (**a**) Nonbuffered formic acid and (**b**) buffered citric acid were injected intradermally to induce itch behavior in the nape skin model (n = 10). $^{**}P < 0.01$ versus saline or pH 7.4. (**c**) Comparison of itch-inducing potency of well-known pruritogens, including DCA, citric acid (pH 3.0), α-methyl-5-HT, chloroquine, and CP48/80 (n = 6–24). (**d**) In cheek assay, algogen capsaicin treatment induced wiping response, and pruritogen chloroquine treatment induced scratching response; citric acid (pH 3.0) injection induced both wiping and scratching response (n = 8). $^{*}P < 0.05$. (**e, f**) Effects of histamine conditioning on citric or formic acid (pH 3.0)-induced scratching in cheek assay (n = 6). $^{*}P < 0.05$. Data are mean ± standard error of the mean. DCA, deoxycholic acid; HT, hydroxytryptamine; M, mol/L.

are well known to sense tissue acidosis (Chen et al., 2014). We pharmacologically and genetically tested whether TRPV1, TRPA1, and ASIC3 are involved in acidic citrate-induced scratching. In wild-type mice, capsazepine (a selective TRPV1 antagonist), HC-030031 (a selective TRPA1 antagonist), or amiloride (the pan-ASIC blocker) was co-injected intradermally with citric acid (pH 3.0) in the nape skin of wild-type mice. Intradermal injection of capsazepine significantly inhibited the acidic citrate-induced scratching (Figure 3a). Capsazepine also inhibited the enhancement of acidic citrate-induced scratching in histamine-sensitized skin (see Supplementary Figure S3 online). In *TrpV1*$^{-/-}$ mice, acidic citrate-induced scratching was, however, impeded in part compared with wild-type controls (Figure 3b). Intradermal HC-030031 had no effect on acidic citrate-induced scratching (Figure 3c); in *TrpA1*$^{-/-}$ mice, acidic citrate-induced scratching was normal (Figure 3d). Intradermal amiloride had no effect on acidic citrate-induced scratching (Figure 3e); in *Asic3*$^{-/-}$ mice, acidic citrate-induced scratching was normal (Figure 3f). From these in vivo studies, we concluded that TRPV1, but not TRPA1 or ASICs, plays an important role in mediating acidic citrate-induced scratching in mice.

TDAG8 is the most abundant proton-sensing GPCR in DRG pruriceptors

Because acidic citrate-induced scratching was partially impeded in *TrpV1*$^{-/-}$ mice, activation of TRPV1 in DRG pruriceptors cannot fully explain the mechanism of acid-induced effect. We wondered whether any of proton-sensing GPCRs is expressed in DRG pruriceptors and involved in acidic citrate-induced scratching. We thus harvested DRG pruriceptors and performed single-cell reverse transcriptase–PCR. A recent study has shown that DRG pruriceptors are selectively located within the voltage-gated sodium channel 1.8 $(Na_V1.8)^+$/isolectin B4 $(IB4)^-$

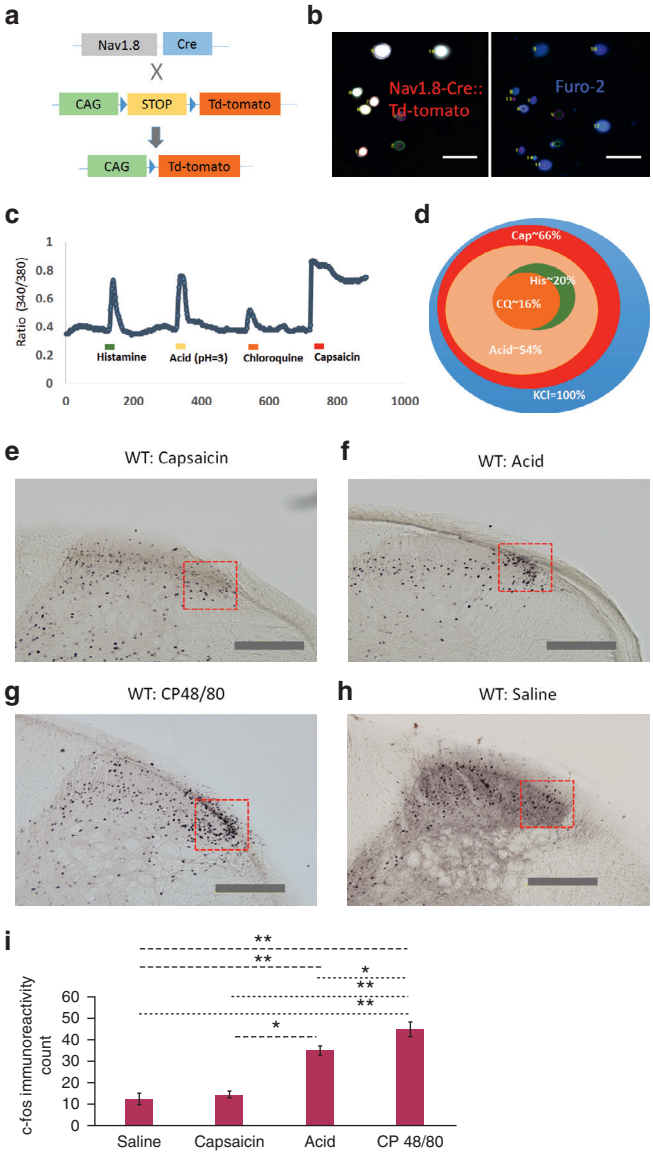

Figure 2. Proton induced calcium response in DRG pruriceptors and generated pruriception-specific c-Fos response in the dorsolateral cervical spinal cord. (**a, b**) Cervical DRG neurons from $Na_V1.8$-Cre::Td-tomato mice were cultured for calcium imaging study. Scale bar = 100 μm. (**c**) A representative result shows elevated calcium level on treatment with histamine, acidic Hanks balanced salt solution (pH 3.0), chloroquine, or capsaicin. (**d**) Proportion of $Na_V1.8$-positive DRG neurons showing response to each treatment (n = 215 from 3 mice). (**e**–**h**) Immunostaining of c-Fos^+ cells responding to capsaicin, citric acid, CP48/80, or saline treatment 30 minutes after intradermal injection. Scale bar = 200 μm. (**i**) To quantify the pruritus-related c-Fos signals in the dorsolateral cervical spinal cord, immunoreactivity in the red dashed-line square in **e**–**h** was calculated by using Image J software (National Institutes of Health, Bethesda, MD) (n = 6). Data are mean ± standard error of the mean. $*P < 0.05$, $**P < 0.01$. CAG, chicken beta-actin gene promoter; Cap, capsaicin; CP, compound; DRG, dorsal root ganglion; His, histamine; WT, wild type.

population and are mainly $Nppb^+$ or GRP^+ (Chiu et al., 2014). We verified the result first with green fluorescence-IB4 staining in the cervical DRG culture from $Na_V1.8$-Cre::Td-tomato mice. Td-$tomato^+$ DRG neurons were divided into $IB4^+$ and $IB4^-$ by the appearance of green fluorescence and harvested for single-cell reverse transcriptase–PCR to detect the expression of TrpV1 and Nppb. The pruriceptor marker Nppb was selectively detected in the $Na_V1.8^+/IB4^-$ population (Figure 4a). Among the 128 $Na_V1.8^+/IB4^-$ neurons collected, 48 (~38%) showed the expression of both TrpV1 and Nppb. No $Nppb^+$ neurons were found in the $Na_V1.8^+/IB4^+$ population. These $Na_V1.8^+/IB4^-$ neurons were processed for detection of OGR1, TDAG8, G2A, and GPR4. Among the 48 $Nppb^+$ DRG pruriceptors, 13 were $OGR1^+$ (~27%), 34 were $TDAG8^+$ (~71%), 10 were $G2A^+$ (~21%), and 3 were $GPR4^+$ (~6%). Our results suggest that TDAG8 is the most abundant proton-sensing GPCR in cervical DRG pruriceptors (Figure 4b).

TDAG8 knockout conferred a selective phenotype in acidic citrate-induced itch

To verify whether TDAG8 plays a role in acidic citrate-induced itch, we screened the scratching behavior induced by citric acid (pH 3.0) in TDAG8-knockout ($TDAG8^{-/-}$) mice. Compared with wild-type mice, $TDAG8^{-/-}$ mice showed significantly reduced acidic citrate-induced scratching response (Figure 4c and d).

$TDAG8^{-/-}$ mice showed normal scratching on challenge with chloroquine, α-methyl-5-HT, or CP48/80 (see Supplementary Figure S4 online). Also, we found that $TDAG8^{-/-}$ mice showed a normal scratching response to bile acid, another pruritogen that plays a role in chronic liver disease and acts on TGR5 to generate an itch sensation. Therefore, the pruriception deficit in $TDAG8^{-/-}$ mice was acid specific.

TDAG8 potentiated acid-induced calcium response by regulating TRPV1

We previously demonstrated that TDAG8 activation by acid (pH 6.4) sensitized TRPV1 response to capsaicin in inflamed DRG neurons (Chen et al., 2009). To test whether TDAG8 also sensitizes the TRPV1 response to acid, we first transfected plasmid TDAG8-pIRES-GFP into HEK293T cells. As expected, TDAG8 mediated acid-induced cAMP accumulation at all pH values tested, with peak response at pH 6.0–6.8. (Figure 5a). Intracellular calcium ($[Ca^{2+}]i$) level was increased by pH value (pH 7.6–5.0) in TDAG8-transfected cells, especially at pH 5.5 and 5.0 (Figure 5b). $[Ca^{2+}]i$ level was increased at pH 5.0 in cells with internal ribosome entry site (IRES)-vector control transfection, so we used pH 5.5 as a standard acid stimulus for further pharmacological study. Increased $[Ca^{2+}]i$ level at pH 5.5 was completely inhibited by removal of extracellular calcium with 2 mmol/L EGTA, so $[Ca^{2+}]i$ content after acid treatment was from calcium channels (Figure 5c and d). Addition of the TRPV1 antagonist capsazepine inhibited increased $[Ca^{2+}]i$ levels after pH 5.5 treatment. Treatment with the pan-ASIC blocker amiloride at 30 μmol/L had no effect under the same conditions (Figure 5d). Acid may activate TDAG8 and increase $[Ca^{2+}]i$ levels via TRPV1, but not via ASICs. With co-transfection of TDAG8 and TRPV1, TDAG8 dose-dependently potentiated the TRPV1-mediated calcium response elicited by acid treatment (Figure 5e). Furthermore, this synergism was completely impeded in the presence of the TRPV1 antagonist capsazepine at 20 μmol/L. Inhibitors for phospholipase-Cβ (U73122) and Gβγ (gallein) significantly inhibited the calcium response elicited by acid stimulation (pH 5.5) of TDAG8/TRPV1 co-expressing cells (Figure 5f). Thus, TDAG8

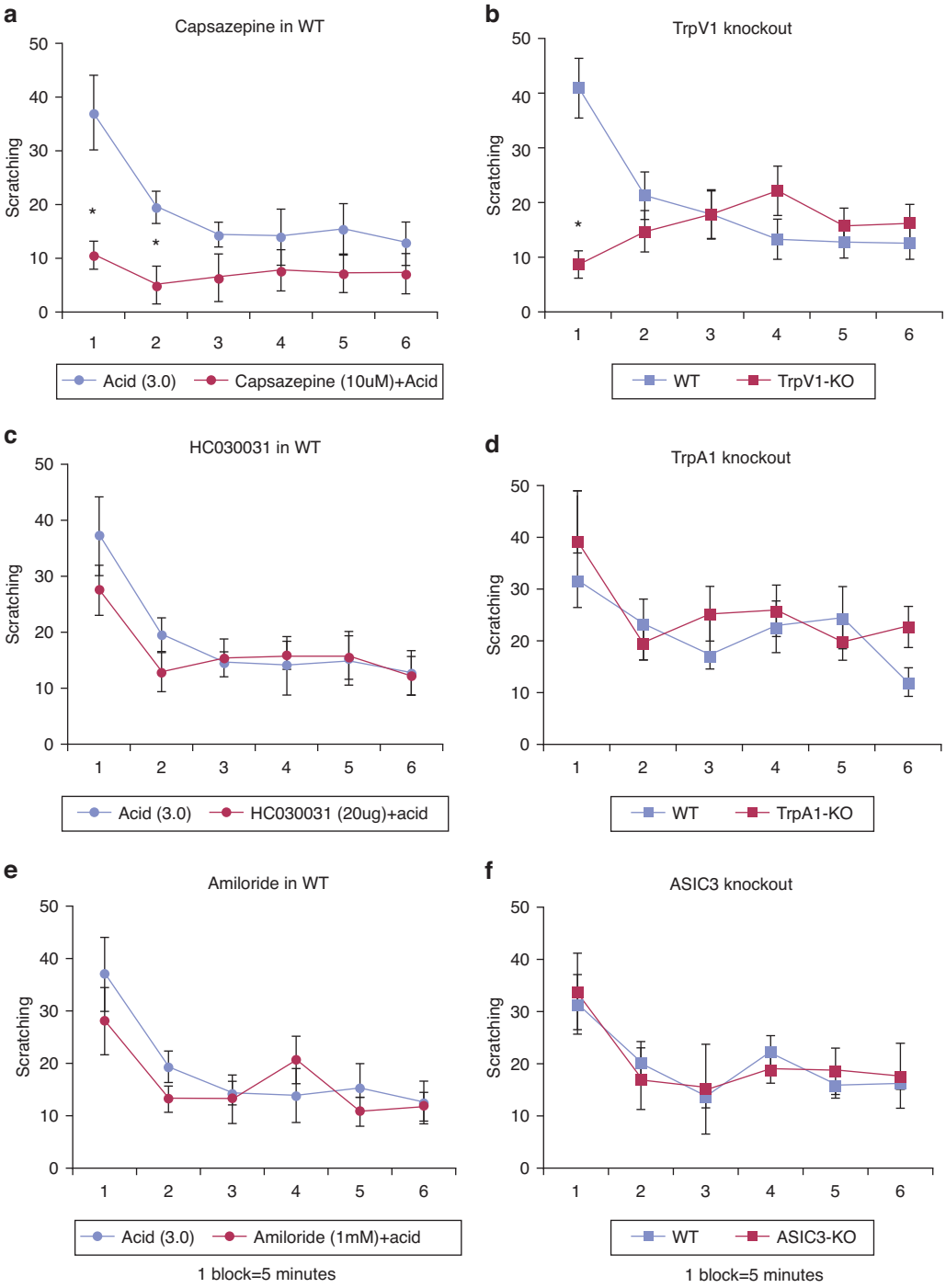

Figure 3. TRPV1 is involved in the acid-induced itch response in mice. (**a**) Citric acid (pH 3.0, 10 μl)-induced scratching response was decreased by intradermal co-injection of capsazepine (10 μmol/L) in nape skin of WT mice. (**b**) Citric acid-induced scratching behavior was partly decreased in *TrpV1*-KO mice. (**c**) Co-injection of HC030031 (20 μg/10 μl, intradermal) in nape skin had no effect on citric acid-induced scratching response in WT mice. (**d**) Citric acid-induced scratching behavior was normal in *TrpA1*-KO mice. (**e**) Co-injection of amiloride (1 mmol/L) in nape skin had no effect on citric acid-induced scratching response in WT mice. (**f**) *Asic3*-KO and WT mice showed no difference in citric acid-induced scratching behavior. n = 8–12. Data are mean ± standard error of the mean. *$P < 0.05$ comparison between groups. ASIC, acid-sensing ion channel; KO, knockout; M, mol/L; WT, wild type.

may mediate acidic citrate-induced itch by sensitizing TRPV1 via phospholipase-Cβ and Gβγ pathways in DRG pruriceptors.

DISCUSSION

In sensitized skin all kinds of nociceptive stimulation, including the most unspecific electrical stimulation, evokes itch (Hosogi et al., 2006; Ikoma et al., 2004). Thus, the activation of nociceptors is essential for the induction of itch in eczema. In normal skin—without pre-existing sensitization—nociceptive stimulation can induce itch when the application is very focal. This is explained by the spatial pattern of discharging nociceptors as the basis of spinal spatial contrast theory (Namer et al., 2008; Namer &

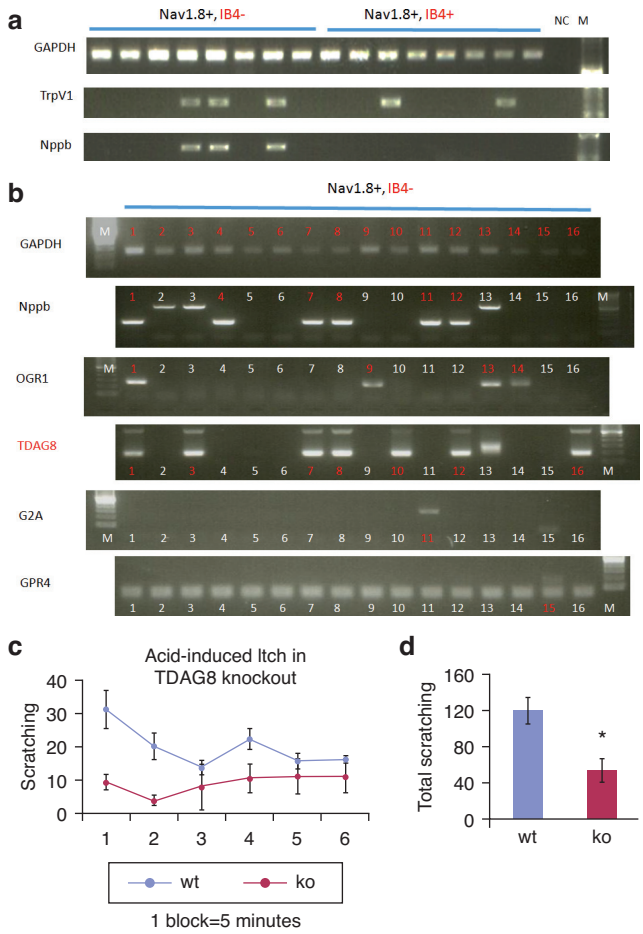

Figure 4. A role for TDAG8 in pruriception. (**a**) Single-cell reverse transcriptase–PCR analyses of $Na_V1.8^+$ DRG neurons. $TRPV1^+$ neurons were detectable in both the $IB4^+$ and $IB4^-$ population, and the DRG pruriceptor marker Nppb was detected only in the $IB4^-$ population. (**b**) To screen the expression of the four proton-sensing GPCRs in the pruriceptors, we used 64 $Na_V1.8^+/IB4^-$ DRG neurons for single-cell reverse transcriptase–PCR to detect Nppb, OGR1, TDAG8, G2A, and GPR4. TDAG8 was the most abundant proton-sensing GPCR (~71%) in $Nppb^+$ DRG pruriceptors. (**c**) Compared with WT mice (n = 6), TDAG8-KO mice (n = 9) showed decreased scratching behaviors after intradermal injection of citric acid in the nape skin model. (**d**) Cumulative scratching response over the 30 minutes after injection. Data are mean ± standard error of the mean. $^*P < 0.05$ versus WT. DRG, dorsal root ganglion; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPCR, G-protein–coupled receptor; IB4, isolectin B4; KO, knockout; M, marker of 100bp DNA ladder; NC, negative control; WT, wild type.

Reeh, 2013; Schmelz, 2010). Here, we provide evidence that acidosis can be one of the causes that evokes itch-like sensation in mouse skin. The acidic citrate-induced itch-like sensation required activation of two proton-sensing receptors, TRPV1 and TDAG8. Especially TDAG8-mediated pruriception was acid-specific, because $TDAG8^{-/-}$ mice showed normal scratching responses to all other pruritogens. TDAG8 is more sensitive to acid than TRPV1. Given that acid is gradually diffused in the injected skin and assumed to simultaneously activate a wide range of sensory afferents, it is hard to explain the results based on the labeled line theory. Instead, similar to itch induced by localized nociceptor activation (Sikand et al., 2009), the observed acidic citrate-induced scratching behaviors may be based on the pattern of discharge, including nociceptors, pruriceptors, and low-threshold afferents being activated by reduction of free calcium. Alternatively, the spatial contrast theory could reconcile the dual roles of acid in itch and pain, because acid could evoke sharp contrast activation of nociceptors with TDAG8-TRPV1 coupling among TDAG8-negative afferents. The interaction of TDAG8 and TRPV1 in itch-like sensation is important under pathological conditions because the number of $TDAG8^+$ (or $TDAG8^+/TRPV1^+$) DRG neurons is increased 24 hours after intraplantar Complete Freund's Adjuvant or carrageenan injection (Chen et al., 2009). Thus, the results of acidic citrate-induced scratching response indicate that a sensitization process that is involved in inflammatory pain could also be operational in itch. Nevertheless, mice have limited types of behavioral responses, and therefore we cannot determine what the mouse is feeling but only observe the responses manifested.

Algogen and pruritogen nature of protons

Almost two decades ago, the discovery of the acid-sensing capacity in DRG sensory neurons led to studies of acid signaling in pain research because tissue acidosis accompanies many painful inflammatory and ischemic conditions (Bevan and Yeats, 1991; Krishtal and Pidoplichko, 1981; Steen et al., 1992). Intradermal infusion of acidic solution-induced pain was reported in humans, with no reduction after repeated capsaicin application; furthermore, co-injection of amiloride (1 mmol/L) inhibited pain induced by intradermal acid (Jones et al., 2004; Ugawa et al., 2002). Although these human studies highlighted the importance of ASICs in modulating the proton-induced pain, the selectivity of amiloride is in doubt because it also blocks TRPA1 (Banke, 2011). Here, we demonstrated that pharmacological antagonism and genetic ablation of TRPV1 but not TRPA1 or ASICs impaired acidic citrate-induced scratching in mice. A recent study suggested that acetic acid (pH 3.5–4.0, nonbuffered) is not a potent pruritogen and that ASIC3 plays a role in acid-mediated potentiation of Ser-Leu-Ile-Gly-Arg-Leu-NH_2 itch (Peng et al., 2015). Our result is consistent with this report because formic acid, without a pH buffer capacity such as acetic acid, induced scratching response only at the extreme pH value of 1.9. Under pH-buffered conditions, 0.2 mol/L of citric acid at pH 4.7 could significantly generate more scratching responses than at pH 7.4. We conclude that under normal conditions, ASICs do not play a role in an acute, acid-induced scratching response in mice. However, we cannot exclude a possible role of TRPA1 in acidic citrate-induced scratching, because human (but not other species) TRPA1 is extremely sensitive to acidosis (de la Roche et al., 2013).

Role of TDAG8 in pruriception

Transcriptome studies of purified neurons indicated that TRPV1, histamine H_1 receptor, and Nppb are enriched in $Na_V1.8^+/IB4^-$ populations and that TRPA1 and MrgprA3 are enriched in $Na_V1.8^+/IB4^+$ populations (Chiu et al., 2014). Among four proton-sensing GPCRs, TDAG8 was the most abundant in $Nppb^+$ DRG pruriceptors in the $Na_V1.8^+/IB4^-$ populations. $TDAG8^{-/-}$ mice showed a deficit to acidic citrate-induced scratching but normal response to other pruritogens including chloroquine, α-methyl-5-HT, CP48/80, and bile acid. In cultured HEK293T cells with proton

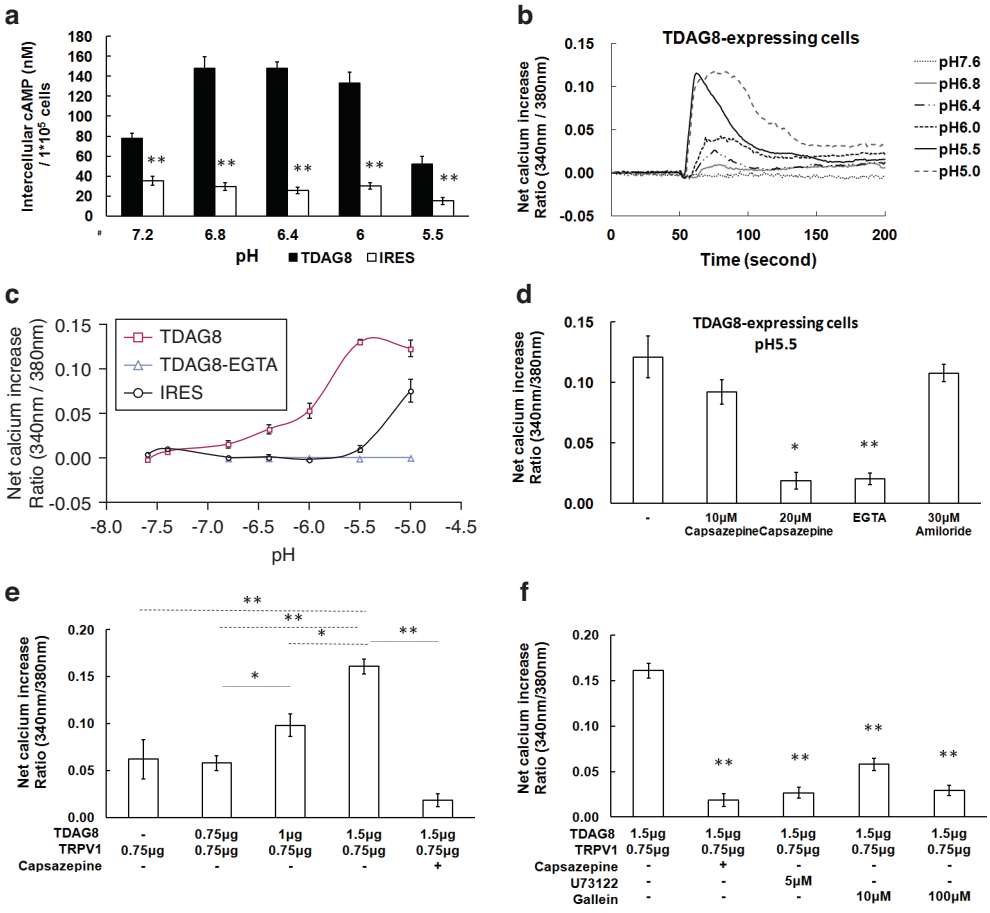

Figure 5. TDAG8 potentiates TRPV1-mediated intracellular calcium in HEK293T cells after proton stimulation. (**a**) The pH-dependent cAMP accumulation in TDAG8-expressing cells or vector (IRES)-expressing cells. (**b**) Time course of $[Ca^{2+}]_i$ signal after the addition of the indicated buffer. (**c**) The pH-dependent curves of net calcium increase in TDAG8-expressing cells in the presence or absence of EGTA (ng/ml) or in IRES-expressing cells in the absence of EGTA. At each pH point, peak values of $[Ca^{2+}]_i$ signals (approximately 20 seconds after the addition of pH buffer) was presented. (**d**) Peak values of net calcium increase with pH 5.5 stimulation with and without capsazepine (10, 20 μmol/L) or amiloride (30 μmol/L). (**e**) Net calcium increase of TDAG8- and/or TRPV1-transfected cells exposed to pH 5.5 with and without capsazepine (20 μmol/L). n = 20–47. (**f**) TDAG8- and/or TRPV1-transfected cells exposed to pH 5.5 with and without U73122 (5 μmol/L) or gallein (10 μmol/L, 100 μmol/L). n = 16–35. Data are mean ± standard error of the mean. $^*P < 0.05$, $^{**}P < 0.01$. IRES, internal ribosome entry site; M, mol/L.

stimulation, TDAG8 potentiated a TRPV1-mediated calcium response via a Gβγ/phospholipase-Cβ pathway. TDAG8 may mediate itch by regulating TRPV1 function. Previous studies also showed that TDAG8 mediated acid-induced response via G-protein s subunit (Gs)/cAMP/protein kinase A or $G_{12/13}$/RhoA pathway (Ihara et al., 2010; Ishii et al., 2005; Mogi et al., 2009). Further studies should determine whether the Gs/protein kinase A or $G_{12/13}$/RhoA signaling is involved in the acidic citrate-induced itch-like sensation in DRG pruriceptors.

CONCLUSION

Acid signaling is polymodal in a somatosensory system and is involved in nociception, pruriception, antinociception, all of which are coupled with distinguishable proton sensors in sensory neurons on the molecular level. Briefly, ASIC3 and TRPV1 are involved in acid-induced nociception and hyperalgesic priming, TRPV1 and TDAG8 are for pruriception, and a non-ASIC3, non-TRPV1 proton-sensing receptor is required for antinociception in muscle afferent neurons (Sun and Chen, 2016). However, a limitation of the current work is the lack of differentiation between the role of specific sensory transduction pathways (e.g., TRPV1) and the role of spatial contrast of activation patterns of single nociceptors. This limitation is commonly ignored in the discussion of the itch and pain field and requires further clarified in future studies.

MATERIALS AND METHODS

Mice

Wild-type C57BL/6JNarl mice were purchased from the National Laboratory Animal Center (Taipei, Taiwan) and used as a backcross pool for all lines of genetically modified mutant mice. Mice with knockout of *TrpV1* and *TrpA1* were obtained from the Jackson Laboratory (Bar Harbor, ME), *Asic3*-knockout mice were generated in our laboratory (Chen et al., 2002), and TDAG8-knockout mice were generated with use of the Sleeping Beauty transposon system (Horie et al., 2003). $Na_V1.8$-Cre mice were obtained from John Wood (Stirling et al., 2005) and crossed with CAG-Td-tomato Cre-reporter mice. For behavioral studies, all mutant mouse lines were congenic after backcrossing to C57BL/6JNarl for at least 10 generations. All experimental procedures with mice (12- to 18-week-old males) were approved by the Institutional Animal Care and Use Committee of Academia Sinica.

Drugs and administration

Chemical compounds including citric acid, sodium citrate, formic acid, capsaicin, chloroquine, amiloride, chlorpheniramine maleate, histamine, and CP48/80 were from Sigma-Aldrich (St. Louis, MO). Capsazepine and HC-030031 were from Tocris (Bristol, UK). α-Methyl-serotonin was from Abcam (Cambridge, UK). Citric acid (0.2 mol/L) at pH 4.7, 3.0, and 1.9 was obtained by mixing pure 0.2-mol/L citric acid and 0.2-mol/L sodium citrate. Formic acid at pH 4.7 (~0.000001%, volume/volume), 3.0 (~0.01%), and 1.9 (~1%) was prepared by sequential dilution of 98% formic acid in sterile saline. To compare the potency of different pruritogens, α-methyl-5-HT

(30 μg/10 μl), chloroquine (200 μg/10 μl), deoxycholic acid (25 μg/10 μl), and CP48/80 (100 μg/10 μ) were freshly prepared, with sterile saline used as a vehicle. To verify the roles of TRPV1, TRPA1, and ASIC3 in acid-induced itch, capsazepine (10 μmol/L), HC-030031 (20 μg), or amiloride (1 mmol/L) was co-injected with 0.2 mol/L of citric acid (pH 3.0) intradermally. Capsaicin (10 μg/10 μl) was dissolved in a vehicle of 7% Tween-80 in saline. Capsazepine and HC-030031 were dissolved in 20% DMSO in saline. Chlorpheniramine (10mg/kg) was intraperitoneally injected 30 minutes before acid injection.

Itch behavioral study

In the mouse cheek and nape skin assay models, mice under anesthesia with isoflurane were shaved at least 24 hours before the experiment. On the testing day, mice were placed individually into a glass chamber (25 × 16 × 18 cm) with approximately 1-cm–thick fresh bedding sawdust (or without bedding in Figure 1e and f) for 30-minute habituation; pruritogen was applied intradermally at a volume of 10 μl with use of a 30-guage needle in a Hamilton 25-μl microsyringe. After drug injection, scratching behavior was recorded for 30 minutes in the same chamber. For all itch behavioral studies, mouse behavior was videotaped and scored by a well-trained observer blinded to the treatment. In both the nape skin and cheek models, *scratching* was defined as when the mouse lifted the hind paw, touched the shaved skin area, and returned the paw to the floor. In the cheek assay, *wiping/digging* was defined as when the mouse wiped the shaved skin on the cheek unilaterally with the forearm or dug into the bedding (Shimada and LaMotte, 2008). For histamine conditioning, acid was injected into cheek skin at 0.5–0.7 cm away from histamine injection site 30 minutes later (see Supplementary Figure S3 online).

Immunohistochemistry

Mice were anesthetized with urethane (1.5 g/kg, intraperitoneal) 30 minutes after the itch-behavioral recording and perfused transcardially with 25 ml of 0.02 mol/L of phosphate buffered saline (PBS) (1 × PBS, pH 7.4, at 4 °C), then 25 ml of cold fixative (4% [weight/volume]) paraformaldehyde and 0.12 mol/L PBS (pH 7.4, at 4 °C). Cervical spinal cord at the level of C1–C6 was dissected and post-fixed in the same fixative at 4 °C for 16 hours; paraformaldehyde-fixed mouse spinal cords were sectioned at 100-μm thick with use of a vibrating tissue slicer (Vibratome 1000 Plus, Rankin Biomedical, Holly, MI). For free-floating staining, slides were first bleached in PBS containing 0.03% H_2O_2 for 30 minutes, blocked in PBST (PBS + 0.1% Triton X-100) containing 5% bovine serum albumin and 5% normal rabbit serum at room temperature for 60 minutes, then incubated with rabbit-anti-c-Fos antibody (1:1000) in blocking solution overnight at 4 °C. Sections were washed three times with PBST and incubated for 1 hour at room temperature with biotinylated goat-anti-rabbit antibodies (1:500). After three PBST washes, sections were incubated in avidin-biotin premix solution (1:1000 in 1 × PBS, Vector Labs, Burlingame, CA), and immunoreactivity signals were visualized by a nickel-diaminobenzidine method. C-Fos immunoreactivity was quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

Calcium imaging

DRGs (C1–C6) were cultured as previously described (Chen et al., 2014). To measure the calcium response to different pruritogens, DRG neurons were washed with 1 × Hanks balanced salt solution and incubated with 5 μmol/L fura-2-acetoxymethylester (Invitrogen, Carlsbad, CA) containing 0.3% bovine serum albumin for 45 minutes at 37 °C. Fluorescence measurements involved use of the Zeiss Axioskop FS upright microscope (Carl Zeiss AG, Oberkochen, Germany) fitted with an ORCA ER CCD camera (Hamamatsu Photonics, Hamamatsu, Japan). Fura-2 was excited at 340 nm and 380 nm (excitation time = 200 or 300 ms) with a rapid-switching monochromator (TILL Photonics, Gräfelfing, Germany), and the emitted fluorescence was filtered with use of a 510-nm longpass filter. Background-subtracted mean fluorescence intensity ratios (F340/F380) were displayed by use of Metafluor software (Molecular Devices, Sunnyvale, CA). The standard bath solution of 1 × Hanks balanced salt solution contained 100 mmol/L NaCl, 2 mmol/L KCl, 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 1 mmol/L $NaHPO_4$, 4.2 mmol/L $NaHCO_3$, 12.5 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 10 mmol/L glucose (~300 mOsm/kg, pH 7.4).

For a heterologous expression system, TDAG8 and TRPV1 cDNAs were cloned into the vector pIRES-hrGFP-2a (pIRES-GFP) and transfected to HEK293T for calcium imaging studies as described (Chen et al., 2009). Briefly, at 17 to 19 hours after transfection, cells were preincubated at 37 °C with serum-free DMEM containing 2 μmol/L of fura-2 acetoxymethyl ester (Invitrogen) for 40 minutes in HEPES/2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (125 mmol/L NaCl, 1 mmol/L KCl, 5 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 8 mmol/L glucose, 10 mmol/L HEPES and 15 mmol/L MES; pH 7.6). After being washed, cells were supplemented with 300 μl HEPES/MES buffer (pH 7.6), then stimulated with the indicated pH of HEPES/MES buffer (600 μ′). For EGTA experiments, 5 mmol/L $CaCl_2$ was removed from HEPES/MES buffer, and 2 mmol/L EGTA was added in the buffer.

Single-cell reverse transcriptase–PCR

Cultured DRG neurons from $Na_V1.8$-Cre::CAG-Td-tomato double transgenic mice were first processed for green fluorescent Alexa Fluor 488-IB4 (Thermo Fisher Scientific, Waltham, MA) staining and then immersed in the chamber filled with artificial cerebrospinal fluid. Sensory neurons were identified by the presence (or absence) of Td-tomato ($Na_V1.8^+$) and green fluorescence ($IB4^+$) under fluorescence microscopy. Single DRG neurons were collected for single-cell RNA isolation and reverse transcriptase–PCR as previously described (Lin et al., 2016). Samples with glyceraldehyde-3-phosphate dehydrogenase signals were selected and processed for two-step nested PCR with intron-spanning primers for TRPV1, Nppb, OGR1, TDAG8, G2A, and GPR4 (see Supplementary Table S1 online).

Statistical analysis

Data are presented as mean ± standard error of the mean. Statistical analysis involved use of SigmaState 3.5 (Systat Software, San Jose, CA). Unless otherwise specified, analyses involved Student *t* test or analysis of variance, with the Holm-Sidak method for post hoc analysis; otherwise, the nonparametric Mann-Whitney test was used. $P < 0.05$ was considered statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

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104-2325-B001-011 to CCC, MOST 104-2320-B-008-001 to WHS); SFI-IvP award; and DEBRA foundation (to MS).

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2016.07.037.

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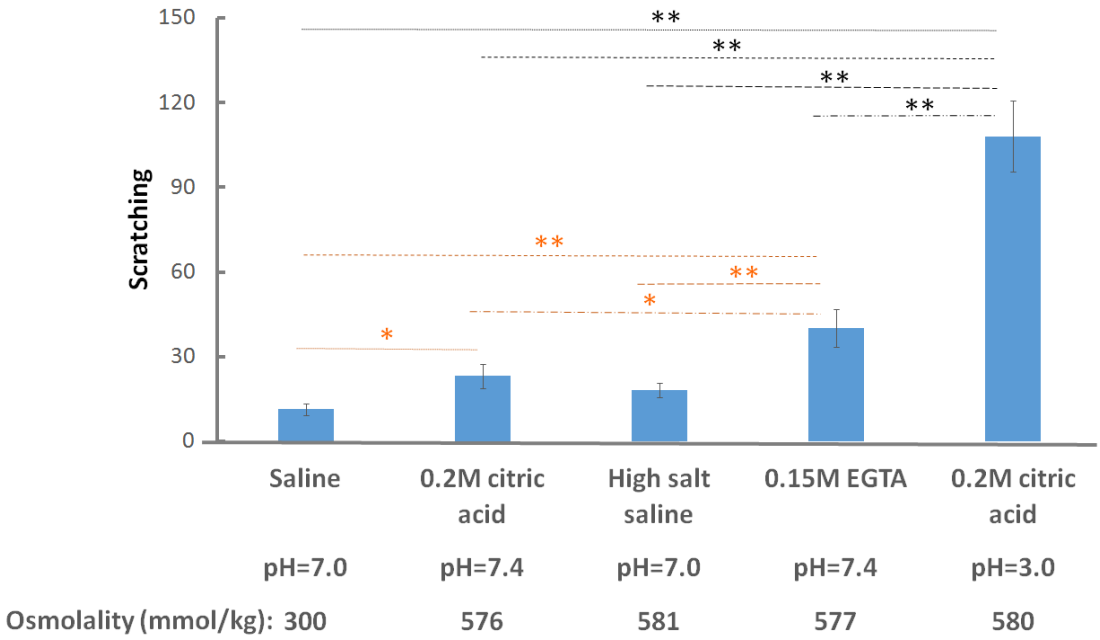

Figure S1. Effect of reduced free calcium on scratching behavior in the nape-skin model. Compared with saline (pH 7.0), 0.2 M citric acid (pH 7.4) would have higher osmolality and lower free calcium. In mice received high-salt saline (pH 7.0, with adjusted osmolality similar to 0.2 M citric acid) showed no change in scratching response as compared with saline. However, saline containing 0.15M EGTA enhanced the scratching responses as compared with saline or high-salt saline group. One-way ANOVA was used and followed by post-hoc Holm-Sidak test to check the difference between groups. N= 5, 9, 8, 8, 10 for saline (pH 7.0), 0.2M citric acid pH7.4, high salt saline, 0.15M EGTA, 0.2M citric acid pH3.0 groups respectively. *P < 0.05, ** P < 0.01. Data are mean ± SEM.

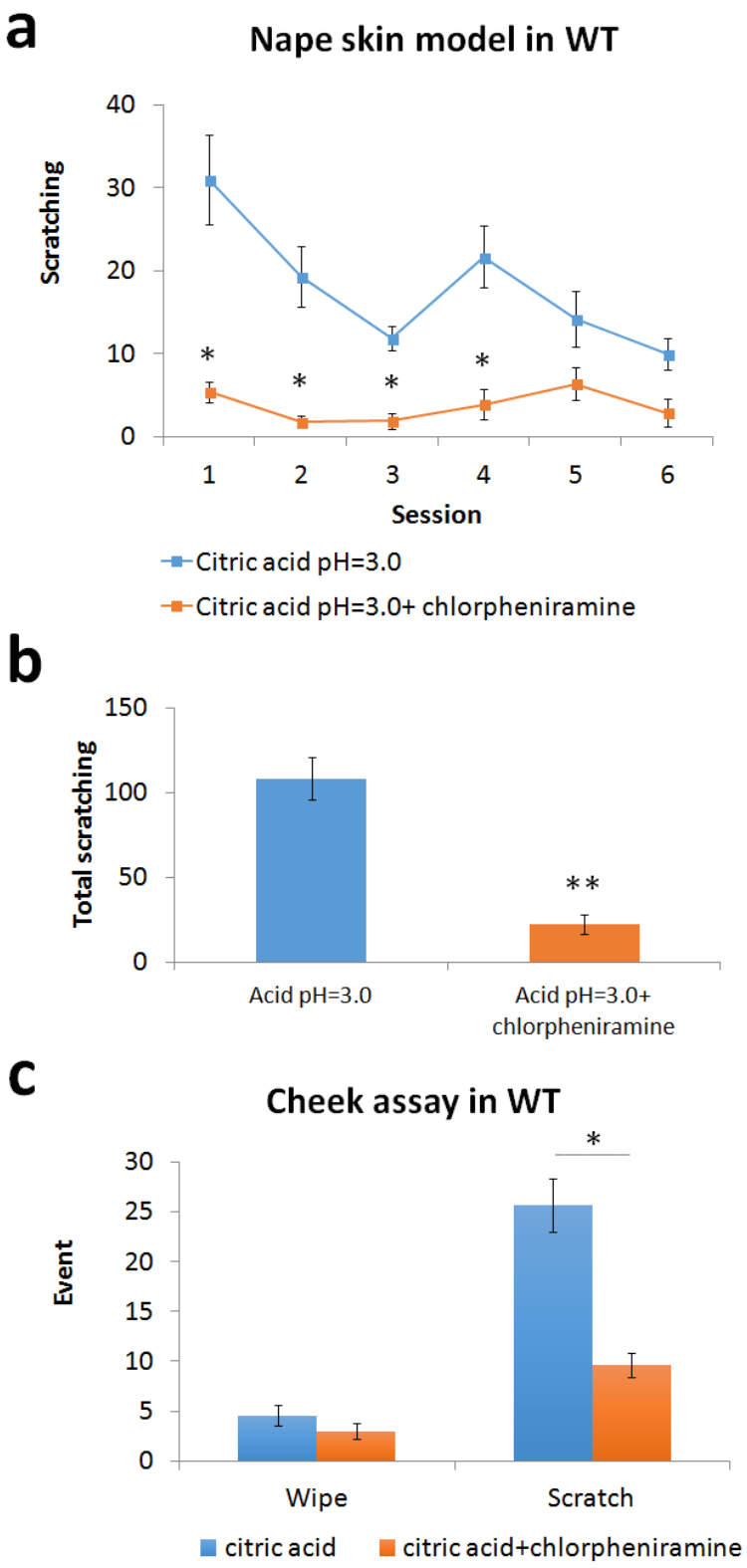

Figure S2. Acid-induced scratching is histamine dependent. (**a,b**) The histamine antagonist chlorpheniramine maleate (10mg/kg, i.p.) inhibits citric acid induced scratching in the nape skin model. N=10, citric acid; N=8, citric acid + chlorpheniramine. (**c**) Chlorpheniramine maleate (10 mg/kg, i.p.) inhibits citric acid-induced scratching in the cheek skin model. N=7, citric acid; N=6, citric acid + chlorpheniramine. *P<0.05, **P<0.01. Data are mean ± SEM.

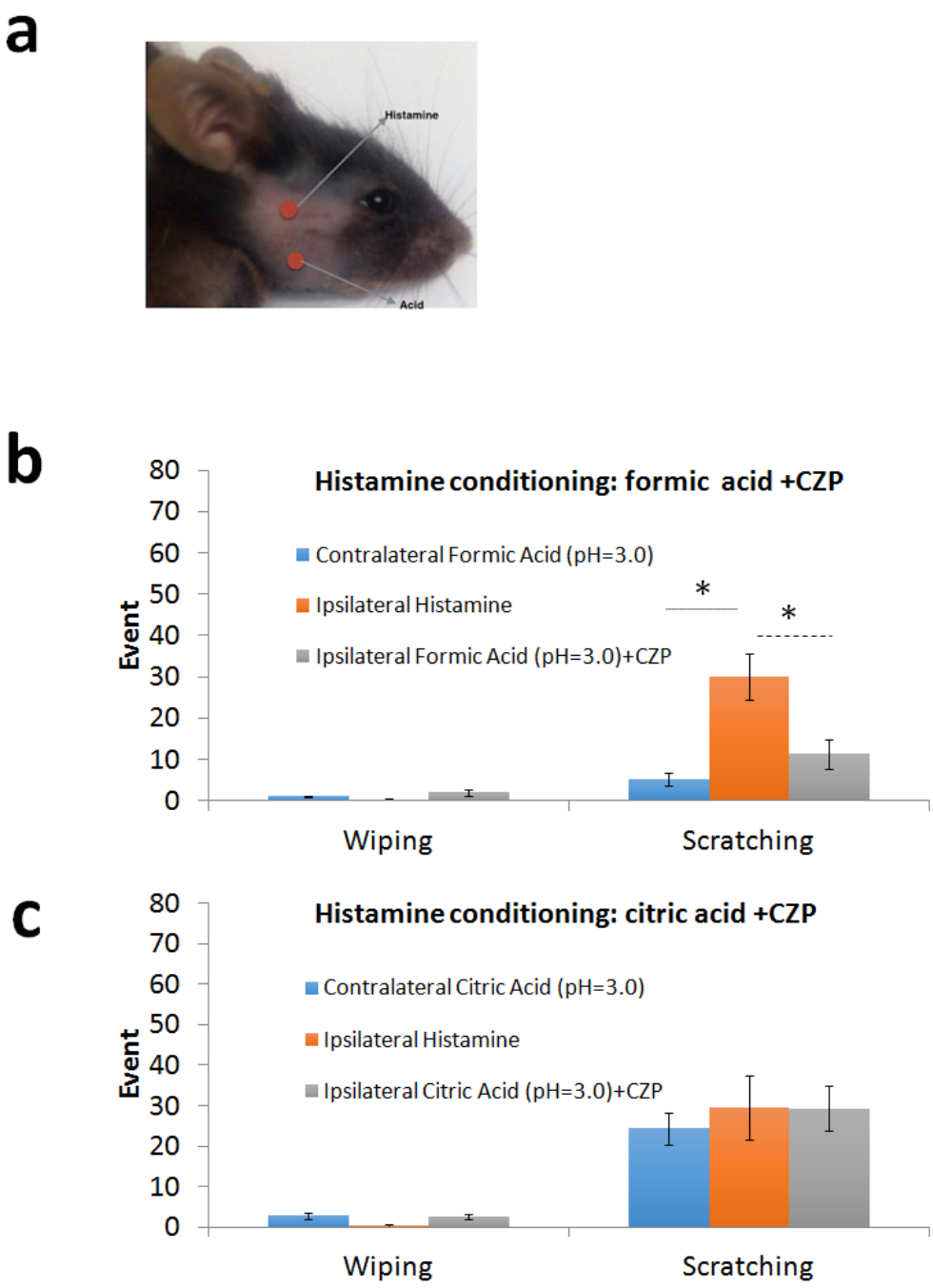

Figure S3. Involvement of TRPV1 in acid-induced scratching in histamine-sensitized skin. (**a**) The experimental design of histamine conditioning study was: (1) injection of citric (or formic) acid to the contralateral side (to histamine) of cheek skin; (2) 30 min later, histamine injection to the ipsilateral side of cheek skin; (3) at 60 min after first acid injection, a second injection of acid (with or without 100 pmole capsazepine [CZP]) to the ipsilateral side at 0.5~0.7 cm away from the histamine injection site. (**b**) Capsazepine inhibited the enhancement of formic acid-induced scratching in histamine-sensitized cheek skin. (**c**) Capsazepine inhibited the enhancement of citric acid-induced scratching in histamine-sensitized cheek skin. *P<0.05. N=6. Data are mean ± SEM.

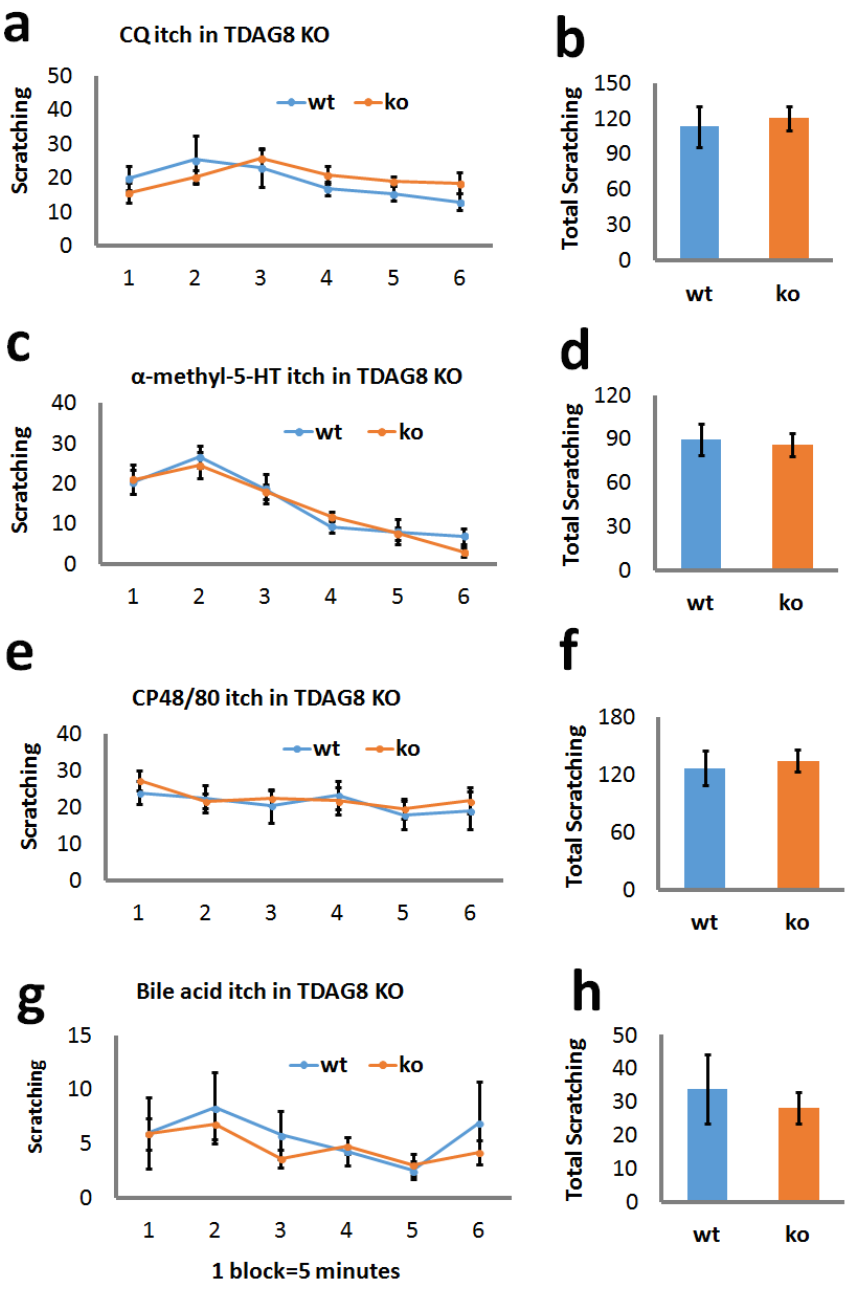

Figure S4. TDAG8-KO mice showed normal itch response to non-proton pruritogens in the nape-skin model. (**a**) TDAG8-KO mice showed normal scratching behavior after intradermal injection of chloroquine (CQ). (**b**) Cumulative scratching response over the 30 min after injection. (**c**) TDAG8-KO mice showed normal scratching behavior after intradermal injection of α-methyl-5-HT. (**d**) Cumulative scratching response over the 30 min after injection. (**e**) TDAG8-KO mice showed normal scratching behavior after intradermal injection of DCA. (**f**) Cumulative scratching response over the 30 min after injection. (**g**) TDAG8-KO mice showed normal scratching behavior after intradermal injection of bile acid. (**h**) Cumulative scratching response over the 30 min after injection. N=10. Data are mean ± SEM.

Table S1. Primers for single-cell RT-PCR

Target Gene	forward primer	reverse primer
TRPV1 outer	5’-TGATCATCTTCACCACGGCTG-3’	5’-CCTTGCGATGGCTGAAGTACA-3’
TRPV1 inner	5’-AAGGCTTGCCCCCCTATAA-3’	5’-CACCAGCATGAACAGTGACTGT-3’
Nppb outer	5’-CAGCTCTTGAAGGACCAAGG-3’	5’-CTTCAAAGGTGGTCCCAGAG-3’
Nppb inner	5’-GTCAGTCGTTTGGGCTGTAAC-3’	5’-AGACCCAGGCAGAGTCAGAA-3’
OGR1 outer	5’-TCTGGCCCAAAGATGGGGAACATCA-3’	5’-AGCCCACGCTGATGTAAATGTTCTC-3’
OGR1 inner	5’-TCTGGCCCAAAGATGGGGAACATCA-3’	5’-GCCAGAAGGGAAGTGAACAG-3’
G2A outer	5’-GGTGACTGCTTACATCTTCTTCTGC-3’	5’-CTGTGTGGATTCTGGACACTTCTTG-3’
G2A inner	5’-GGTGACTGCTTACATCTTCTTCTGC-3’	5’-GGTGAAACGCAGGTAGTGGT-3’
TDAG8 outer	5’-CAAGAGAAGCATCCCTCCAG-3’	5’-AGTACAGAATGGGATCGGCAACACA-3’
TDAG8 inner	5’-TGGACTTTCTCTCCCACCTTGTGCA-3’	5’-CCAGATGGAGAGGCTGGTAA-3’
GPR4 outer	5’-ATATCAGCATCGCCTTCCTGTGCTG-3’	5’-CAGCCACACAATTGAGGCTGGTGAA-3’
GPR4 inner	5’-GGATGAATCTGTACCGCGTC-3’	5’-CAGCCACACAATTGAGGCTGGTGAA-3’
mGAPDH	5'-GGAGCCAAACGGGTCATCATCTC-3'	5'-GAGGGGCCATCCACAGTCTTCT-3'