FXYD6 promotes thermal nociception by regulating TRPV1

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Abstract
FXYD6, an unnecessary auxiliary subunit of Na⁺,K⁺-ATPase, is expressed in the nervous system. However, its functions remain largely unclear. In the present study, we find that FXYD6 is involved in the thermal nociception. FXYD6 was mainly expressed in small-diameter DRG neurons expressing transient receptor potential channel V1 (TRPV1). In the SNS-Cre/Fxyd6flo/flo mice, loss of FXYD6 in these sensory neurons impaired the behavioral responses to noxious heat stimulus and intraplantar injection of capsaicin. The capsaicin-induced and TRPV1-mediated currents were decreased in the FXYD6-deficient DRG neurons. Heterologous expression of FXYD6 could increase the TRPV1 capsaicin-sensitive currents in HEK293 cells. Furthermore, we found that the negatively charged PGDEE motif in C-terminal of FXYD6 is required for the FXYD6/TRPV1 interaction and FXYD6-mediated enhancement of TRPV1. Disrupting the FXYD6/TRPV1 interaction with the TAT-PGDEE peptide could elevate the threshold of thermal nociception. Therefore, FXYD6 maintains the thermal nociception via interacting with TRPV1 channel in nociceptors.

Keywords
FXYD6, TRPV1, DRG, thermal nociception

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Introduction
FXYD6 (Phosphohippolipin), was firstly found in the hippocampus and cerebellum.1,2 FXYD6 belongs to the single-span membrane protein FXYD family.3 FXYD family members are tissue-specific and subunit isoform-specific regulators of Na⁺⁺,K⁺⁺-ATPase (NKA).4,5 As an unnecessary auxiliary subunit of NKA, FXYD6 is found to decrease the NKA activity.6 According to previous studies, FXYD6 is related to diseases such as pancreatic cancer, cholangiocarcinoma and hepatocellular carcinoma.7-10 It is thought to be a candidate gene of schizophrenia, since the human FXYD6 gene is located in Chr11 q23.3 and contains six SNPs related to the schizophrenia.11-13 Furthermore, FXYD6 is expressed in type II spiral ganglion neurons, type II taste bud cells and cochlear stria vascularis of inner ear, and maybe involved in gustation, alcohol addiction, hearing and endolymph homeostasis.14-17 In addition, one study shows that Fxyd6 is widely distributed in almost all subnuclei of parabrachial nucleus, a
relay center for sensory information. Therefore, FXYD6 is a widely distributed molecule with varied physiological activities.

Nociception is the protective response of bodies to harmful stimuli. The DRG neurons are pseudo-physiological activities.

FXYD6 is a widely distributed molecule with varied channel role in thermal nociception by interacting with TRPV1. Our study revealed that FXYD6 played an important role in thermal nociception by interacting with TRPV1. FXYD6 could elevate the threshold of thermal nociception. Therefore, blocking the FXYD6-TRPV1 interaction could regulate TRPV1 in the DRG neurons. The C-terminal PGDEE motif of FXYD6 was required for the FXYD6/TRPV1 interaction and FXYD6-mediated enhancement of TRPV1. FXYD6 could increase the capsaicin-sensitive currents via TRPV1 in the DRG neurons. Furthermore, blocking the FXYD6-TRPV1 interaction elevated the threshold of thermal nociception. Therefore, our study revealed that FXYD6 played an important role in thermal nociception by interacting with TRPV1 channel.

Materials and methods

Animals

Experiments were performed according to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain and were approved by the Committee of Use of Laboratory Animals and Common facility, Institute of Neuroscience, Chinese Academy of Sciences. C57BL/6J mice purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China). Fxyd6 floxed mice (Fxyd6loxP/loxP) were generated by adding two loxP sites between upstream of the Fxyd6 exon 3 and downstream of exon 5. The Fxyd6 gene was deleted selectively in DRG neurons by crossing Fxyd6loxP/loxP mice with BAC transgenic mice expressing Cre recombinase controlled by promoter elements of the Na1.8 gene, which is mainly expressed in small DRG neurons (SNS-Cre). Fxyd6 cKO mice were viable and fertile. Mice were raised together with littermates in pathogen-free environment and their health status was routinely checked. No more than 6 mice were housed in one cage. All animals were housed under a 12-h light/dark cycle at 22–26°C, with ad libitum access to water and chow. Experiments were conducted during the light phase of the cycle. 2- to 4-month-old male mice were used for all in vivo and in vitro experiments.

Genotyping

The Fxyd6 cKO mice were identified by genotyping. A small piece of mouse tail or ear was digested in Proteinase K buffer. The genomic DNA was extracted with phenol/chloroform, precipitated with isopropanol, washed with 75% ethanol and dissolved in water. The mouse genotype was then identified by PCR. The primers for the identification of Fxyd6loxP were 5'-ATTCTGGCCCATTCAGACATTAGG-3' and 5'-CAAAGGGGAACTGAGACCAA-3'. The primers for the identification of SNS-Cre were 5'-ATTTGCCTGCTACCCTGC-3' and 5'-GCATCAAGCTTTCTTTTCGG-3'. Fxyd6 cKO male mice carrying Fxyd6loxP and SNS-Cre (SNS-Cre+/Fxyd6loxP, Fxyd6SNS) were used as the experimental group, while the male mice carrying Fxyd6loxP without SNS-Cre (SNS-Cre+/Fxyd6loxP) were used as the control group.

Plasmid construction

Mouse Fxyd6 and Fxyd6 mutants were subcloned into pcDNA3.1myc-His(+)A vector for Co-IP. Mouse Fxyd6 was subcloned into pIREs2-EGFP vector for electrophysiological recording. Mouse Trpv1 and mCherry sequences were subcloned and fused into pcDNA3.1-His(+)A vector (Myc-tag deleted) for mouse and electrophysiological recording. FXYD6-myc was constructed by PCR amplified the full length cDNAs of mouse FXYD6 with the following 5' and 3' primers: 5'-CCAAGCTTGGCCACCATGGAGACGGTGCTGTC-3' and 5'-CCGGAATTCGTTCTCTGCCTTCTGGGGCTCCG-3'. The primers for the identification of SNS-Cre were 5'-ATTGCCTGCTACCCTGC-3' and 5'-GCATCAAGCTTTCTTTTCGG-3'. The FXYD6 mutants were constructed with KOD and DPNI enzyme. KOD enzyme as a high fidelity enzyme amplified the whole plasmid. DPNI enzyme was used to cleave the methylated plasmid from the E.coli and the mutated plasmid were left. FXYD6 mutant-Myc plasmids were constructed by PCR mutated the different regions of FXYD6-myc with the following 5' and 3' primers: FXYD6 PGDEEmut-Myc: 5'-GCAGCTGCCAGGAGTTGAGACCCACATGGAGACGGTGCTGTC-3' and 5'-AGCTGCGGCCAGCCCACACGACTTCATC-3'. The FXYD6 mutants were constructed with KOD and DPNI enzyme. KOD enzyme as a high fidelity enzyme amplified the whole plasmid. DPNI enzyme was used to cleave the methylated plasmid from the E.coli and the mutated plasmid were left. FXYD6 mutant-Myc plasmids were constructed by PCR mutated the different regions of FXYD6-myc with the following 5' and 3' primers: FXYD6 PGDEEmut-Myc: 5'-GCAGCTGGACCCAGGAGTTGAGACCCACATGGAGACGGTGCTGTC-3' and 5'-AGCTGCGGCCAGCCCACACGACTTCATC-3'.
CCTTTTCTTCTCCTTCAGCTGC-3’; FXYD6 (PFXYDY+PGDEEmut-Myc plasmid was constructed by PCR mutated the PGDEE region of FXYD6 PFXYDYmut-Myc with the following 5’ and 3’ primers: 5’-GGCGGCTGCCGTTGAGAAAATCTCATC-3’ and 5’-AGCTCAGGCAACCCTTGGGCTTCTG ATTGAAACT-3’, and cloned into pcDNA3.1-His(+). A vector. TRPV1-Flag-p2A-mCherry was constructed as following. Firstly, TRPV1-Flag and mCherry sequences were amplified by PCR with the primers for TRPV1-Flag: 5’-ATGGAGAAATGGGCTAGCTTAGACT-3’, and 5’-AGTTAGTACCTCCGCTTCTGTCGTC GTCATCCTTGATGC-3’, and the primers for mCherry: 5’-GGAGAGAACATCTGACCTATGG TGAGCAAGGCAGAAA-3’ and 5’-AGCTAG GCACTTTCCCTATGGGCTGAGAATCCAAGCTTA AC-3’. p2A sequence was obtained from the denatured and annealed primers: 5’-GGAAAGCGGAGC TACTAATTCAGCTGCAAGCGAGCTGGAG ACCTGGAGAGAAACCCTTGGGCTTCTGTC GTCATCCTTGATGC-3’. Next, pcDNA3.1-His(+)A vector was linearized by PCR amplified. Then TRPV1-Flag, mCherry and p2A sequences were fused into pcDNA3.1-His(+)A myc deletion vector by Hieff Clone Enzyme (Hieff Clone™ Plus One Step Cloning Kit).

**Cell culture and transfection**

HEK293T cells and HEK293 cells were cultured in MEM with 10% fetal bovine serum. The cells were transiently transfected with plasmids using Lipofectamine 2000 reagent (Invitrogen) and were used for the following various experiments 24-48 hr after transfection. For co-immunoprecipitation, FXYD6-Myc, as well as FXYD6 mutants PGDEEmut-Myc, PFXYDYmut-Myc and (PFXYDY+PGDEEmut-Myc were co-transfected with TRPV1-Flag-p2A-mCherry into HEK293T cells. For electrophysiological recording, FXYD6-EGFP was co-transfected with TRPV1-Flag-p2A-mCherry into HEK293 cells.

**Preparation of FXYD6 antibodies**

A peptide (CCSFNQKRAPGDEEAQVENLITTN AAEPQKAEN) mapping to the C terminus of mouse FXYD6 was prepared and mixed with Freund’s complete adjuvant, and then used to immunize New Zealand rabbits. The immunizations were repeated 3 weeks and 5 weeks later. The sera were taken and stored at –70°C. The antibody specificity was verified by a pre-absorption assay with peptide at 10⁻⁵ or 10⁻⁶ M, and the DRG tissue samples of Fxyd6 cKO mice. The antibody efficiency was validated in HEK293T cells transfected with FXYD6 plasmids.

**In situ hybridization**

Lumbar DRGs were dissected from adult male mice and processed for ISH. The primers for the probes for Fxyd6 mRNA were 5’-CAGTCCCCGTGCAAGACG-3’ and 5’-GGAGATGGAGGACCTGCAAG-3’, the primers for Il13ra mRNA were 5’-AGGAAGGTTGGTGAGTTCATG-3’ and 5’-GCAGGAAGCTGACAGG-3’, the primers for Mrgprd mRNA were 5’-CAAGCAGACTCCCTCAGACCTAG-3’ and 5’-GAAGGAGGGTGTAAGGGTTAG-3’, the primers for Th mRNA were 5’-AAATTGCTACCTGGGAAGGAGGT-3’ and 5’-GGGGTGTTACCCTATGTGAGTACGTGC-3’. For ISH, cRNA riboprobes were labeled with DIG. Fresh sections of DRG were fixed with 4% paraformaldehyde in DEPC-PBS for 20 min, then acetylated and prehybridized in hybridization buffer for 3 h at 67°C. The sections were then incubated with the hybridization buffer containing 1 µg/ml of the antisense probe for 16 h at 67°C. After hybridization, the sections were incubated in alkaline phosphatase-conjugated sheep anti-DIG antibodies (1:2000; Roche Molecular Biochemicals). The slides were developed in a solution of 1 µl/ml NBT and 3.5 µl/ml BCIP in alkaline phosphatase buffer.

For ISH combined with immunostaining, the hybridized sections were incubated with anti-digoxigenin-AP (1:1000) overnight at 4°C. After being washed in TNT (TS7.5, 0.05% Tween 20) and TS8.0 (0.1 M Tris-HCl, pH 8.0, 0.1 M NaCl, and 10 mM MgCl₂), the sections were incubated with HNPP/FR (1:1:100) in TS8.0. Once the signals were generated, the sections were washed and then processed for immunostaining.

For double fluorescent ISH, one probe was labeled with fluorescein (FITC), whereas the other was labeled with DIG. Both probes were added to the hybridization buffer. After washing and blocking, the sections were incubated with anti-FITC-HRP (1:4000) overnight at 4°C. After washing with TNT buffer, the sections were incubated with TSA-Plus (DNP, 1:100) for 10 min. Then, the sections were washed with TNT and incubated with anti-DIG-AP (1:1000) and anti-DNP Alexa488 (1:500) in 1% blocking reagent overnight at 4°C. The sections were washed with TNT and TS8.0, and then developed with HNPP/FR (1:1:100) in TS8.0. For ISH combined with immunostaining, the hybridized sections were incubated with anti-digoxigenin-AP (1:1000) overnight at 4°C. After being washed in TNT (TS7.5, 0.05% Tween 20) and TS8.0 (0.1 M Tris-HCl, pH 8.0, 0.1 M NaCl, and 10 mM MgCl₂), the sections were incubated with HNPP/FR (1:1:100) in TS8.0. The slides were developed in a solution of 1 µl/ml NBT and 3.5 µl/ml BCIP in alkaline phosphatase buffer.

**Immunostaining**

Adult male mice were fixed with 4% paraformaldehyde and 1% picric acid. L4 and L5 DRGs were dissected and post-fixed for 2 h. Then the tissues were dehydrated and embedded for cryostat sectioning. Cryostat sections of
DRGs were processed for double-immunofluorescent staining or ISH combined with immunostaining. The cryostat sections were incubated in primary antibodies at 4°C overnight, and then incubated in secondary antibodies at 37°C for 45 min.

The primary antibodies against FXYD6 (1:2000, Rb, this paper), TRPV1 (1:500, Go, Santa Cruz, sc-12498), TH (1:1000, Sh, Merk/Millipore, AB1542), NF200 (1:2000, Mo, Sigma, N2912), CGRP (1:500, Go, AbD, 1720–9007), Peripherin (1:500, Rb, Chemicon, AB1530), and Nav1.8 (1:1000, Rb, Alomone Labs, ASC-016). The secondary antibodies were FITC, Cy3 or Cy5-conjugated donkey against goat, rabbit, Mo, or sheep antibodies (1:100; Jackson ImmunoResearch). To label IB4-positive neurons, cryostat sections of DRG were first incubated with 5 μg/ml IB4 (Vector Laboratories), and then were stained with goat antibodies against GSL antibodies (1:1000, Go, Vector Lab, AS-2104), followed by FITC- and Nav1.8 (1:1000, Rb, Alomone Labs, ASC-016). The antibodies against FXYD6 (1:2000, Rb, Proteintech, 16286–1-AP), and Myc (1:2000, Rb, Sigma, C3956) were used.

**Electrophysiological recording**

Whole-cell voltage-clamp recordings were performed to record the capsaicin-induced currents in dissociated small DRG neurons and transfected HEK293 cells. L4 and L5 DRGs of mice were dissected and digested with collagenase (1 mg/ml), trypsin (0.4 mg/ml) and DNase (0.1 mg/ml) in DMEM for 30 min at 37°C. After trituration, freshly dissociated DRG neurons were plated on coverslips and incubated in the ECS (150 mM NaCl, 5 mM KCl, 5 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, 10 mM D-glucose, pH 7.34) for at least 1 h. Transfected HEK293 cells were plated on coverslips and could be identified by EGFP and mCherry fluorescence. Only one neuron or one HEK293 cell was tested per coverslip. Capsaicin was used as agonist of TRPV1.

The whole-cell configuration was obtained in voltage-clamp mode. In brief, we filled a glass electrode (resistance of 3–6 MΩ) with intracellular solution and advanced it quickly to the target depth under moderate positive pressure. The pipette solution contained (in mM) 135 K-gluconate, 0.5 CaCl2, 2 MgCl2, 5 KCl, 5 EGTA, 5 HEPES and 5 D-glucose. When the tip of the electrode was close to a neuron, the tip resistance was increased. We removed the positive pressure and applied negative pressure to aid in seal formation. We achieved the whole-cell configuration using brief bursts of negative pressure to rupture the cell membrane. For the recording of capsaicin-induced currents, dissociated small DRG neurons and HEK293 cells were held at −60 mV in voltage-clamp mode. The translucent and brilliant small DRG neurons and transfected HEK293 cells containing EGFP and mCherry fluorescence were patched and recorded. Whole-cell recordings were performed using the Axopatch 200B amplifier, digitized using the Digidata 1440A interface, and controlled using pCLAMP10.1 software. Inward currents induced by capsaicin were recorded and analyzed. Statistical comparisons were made using unpaired Student’s t test and differences were considered significant at \( p < 0.05 \).

**Preparation of single DRG neurons and single-cell PCR**

After electrophysiological recording, the individual DRG neurons were gently transferred into lysis buffer and processed for single-cell RNA extraction and reverse transcription within 1 h and were subjected to cDNA amplification, according to the manufacturer’s protocol (Superscript III, Invitrogen). Subsequently, cDNA was used in separate PCR reactions. The reaction mixture (20 μl) was amplified using ExTaq (Takara) for 45 cycles. Then, we performed PCR to detect the gene expression level of Fxyd6, Trpv1 and Gapdh, as well as cell type markers, including Gal, Sst, Th, Mrgpra3, and Mrgprd. The PCR products were analyzed on
The cut-off times at 52°C were 5 s at signs of nociception, such as jumping or licking of a paw. The response latency was determined by observing the movement of the hindpaw after the movement of the hindpaw. The latency was measured as the duration from the beginning of the heat stimulus to the occurrence of a hindpaw withdrawal reflex. The cut-off time was 20 s.

In the von Frey test, the mice were habituated in plastic chambers on a mesh floor. Mechanical stimuli were applied using ascending graded individual monofilaments. A von Frey filament was applied 5 times to each testing site of the hindpaw. The bending force of the von Frey filament to evoke paw withdrawal with over 50% occurrence frequency was determined as the mechanical threshold.

Capsaicin solution was injected into the left hindpaw of mice. The licking and lifting time of the injected hindpaw during 10 min after injection was recorded and analyzed.

PGDEE Scramble sequence was obtained from the online website http://www.mimotopes.com/peptideLibraryScreening.asp?id=97. HIV-1 TAT core sequence (YGRKKRRQRRR) is commonly used to help the target protein to penetrate the membrane. HIV-1 TAT-PGDEE (YGRKKRRQRRRPGDEE) and TAT-Scramble (YGRKKRRQRRRREGDPE) were synthesized in GL Biochem (Shanghai) Ltd and then were dissolved well in double-distilled water as stocks (100 mM). In the hotplate test, the mice were habituated and measured the basal threshold. Then the mice were held and intrathecally injected with 10 μl saline or TAT peptide diluted in saline (containing 3 μg peptide). After recovery, the mice were measured the thermal latency from 1 hr to 8 hrs after intrathecal injection.

Statistical analysis

The data are presented as mean ± SEM. Sample number (n) values are indicated in figures, figure legends or results section. Two groups were compared by a two-tailed, unpaired Student’s t test. Comparisons among multiple groups were performed by using two-way ANOVA with Bonferroni’s post hoc test. Statistical analysis was performed using PRISM (GraphPad Software). Differences were considered significant at p < 0.05 (∗p < 0.05, ∗∗p < 0.01, ∗∗∗p < 0.001 and N.S. not significant).

Results

Fxyd6 is mainly expressed in small-diameter DRG neurons

Analysis on our scRNA-seq data showed that Fxyd6 was mainly expressed in the C1 type DRG neurons marked by Gal and the C3 type neurons marked by Th (Figure 1(a)). Fxyd6 was present in about 23.5% of DRG neurons, and they were small-diameter DRG neurons (cross-sectional area of neuron profiles < 600 μm²) (Figure 1(b) and (c)). Double in situ hybridization (ISH) or ISH combined with immunostaining showed that in the lumbar 4 and 5 DRGs of mice, almost all Fxyd6 expressed in all Th-positive neurons and in peripherin-positive (a classical marker of most small-diameter DRG neurons) neurons, but not co-expressed with marker gene Ib31ra or Mrgprd for C2 or C5 types of

Behavioral tests

All experiments were carried out in accordance with the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain, and approved by the Committee of Use of Laboratory Animals and Common facility, Institute of Neuroscience, CAS.

Adult male animals were housed under a 12:12 h light/dark cycle at 22–26°C, and tests were performed blind to genotype. In the Hargreaves and hot plate tests, the response latency to nociceptive stimuli was measured. In von Frey test, the threshold of nociceptive responses was measured. The tests above were stopped at a cut-off time or force for animal protection.

To examine heat sensitivity of the hindpaw, the mice were placed and habituated in plastic chambers on the surface of a glass plate. The radiant light was applied to one of their hindpaws using a radiant heat stimulator (Ugo Basile, 37,370 Plantar Test, Hargreaves Apparatus). The radiant light was applied when the mice were resting quietly and was stopped immediately after the movement of the hindpaw. The latency was determined as the duration from the beginning of the heat stimulus to the occurrence of a hindpaw withdrawal reflex. The cut-off time was 20 s.

The hotplate test was performed by using a hotplate (Ugo Basile, 35,100 Hot/Cold plate) maintained at 52°C. The response latency was determined by observing the signs of noiception, such as jumping or licking of a paw. The cut-off times at 52°C was 45 s.

In the von Frey test, the mice were habituated in plastic chambers on a mesh floor. Mechanical stimuli were applied using ascending graded individual monofilaments. A von Frey filament was applied 5 times to...
small DRG neurons, respectively. Double-immunostaining showed that FXYD6 was mainly distributed in the GAL or TH-positive small DRG neurons, and a few IB4-positive small DRG neurons, but not in NF200-positive large DRG neurons (Figure 2).

**FXYD6 protein is transported to the afferent fibers in the superficial laminae of the spinal cord**

The expression profile of FXYD6 in the nervous systems of adult mice was also examined, and immunoblotting showed that FXYD6 was widely distributed in the hippocampus and cerebellum as reported previously, as while as in the DRG and spinal cord (Figure 3(a)). However, in the lumbar 4 and 5 segments of spinal cord, ISH showed that Fxyd6 was not detected in the superficial laminae (Figure 3(b)). Double-immunostaining showed that in the lamina I and II of spinal cord, FXYD6 was present in the nerve fibers positive for neuropeptides substance P (SP) and CGRP, two classical markers of the superficial laminae of the spinal dorsal horn. Moreover, FXYD6 was not found in the PKCε-containing local neurons of spinal dorsal horn (Figure 3(c)). Thus, FXYD6 is predominantly expressed in the small-diameter DRG neurons, and then could be transported to their afferent fibers in the superficial laminae of the spinal cord.

**Fxyd6 cKO mice have impaired behavioral responses to noxious heat**

Given that FXYD6 is expressed in C1 type of small DRG neurons and present in their afferent fibers in the superficial layers of spinal cord, FXYD6 might play a potential role in the somatosensory regulation. To examine the FXYD6 function, we generated Fxyd6 floxed mice (Fxyd6<sup>F/F</sup>), in which two loxP sites were adding between upstream of the Fxyd6 exon 3 and downstream of exon 5. Because Na<sub>1.8</sub> (SNS, encoded by gene Scn10a)-positive neurons could cover the Fxyd6-positive neurons, we
crossed the SNS-Cre mice with the Fxyd6<sup>F/F</sup> mice to obtain the Fxyd6 cKO mice, namely SNS-Cre/Fxyd6<sup>F/F</sup> (Fxyd6<sup>SNS</sup>) mice (Figures 1(a) and 4(a)). Immunoblotting results showed that FXYD6 level was decreased in Fxyd6<sup>SNS</sup> mice (Figure 4(b)). Then we carried out a series of behavior tests to study the possible roles of FXYD6 in the somatosensory neurotransmission.

In the Hargreaves test of thermal nociception, Fxyd6 cKO mice showed elevated paw withdrawal latency,
compared with control mice (t = 5.006, df = 10, p = 0.0005) (Figure 4(c)). In the hotplate test, Fxyd6 cKO mice also showed elevated paw withdrawal latency at 52°C (t = 2.503, df = 8, p = 0.0367) (Figure 4(c)). However, Fxyd6 cKO mice displayed the comparable mechanical threshold in von Frey test (t = 1.224, df = 10, p = 0.2489) and the normal sensitivity to noxious mechanical stimuli in pinprick test (t = 0.8452, df = 10, p = 0.4178) (Figure 4(c)). Taken together, these results indicated that FXYD6 in DRG neurons could regulate the thermal nociception.

**FXYD6 co-expresses and interacts with TRPV1**

We further studied the underlying mechanism for the FXYD6-mediated regulation of thermal nociception. We performed intraplantar injection of capsaicin or saline into the hindpaw of Fxyd6 cKO mice and control mice, and recorded the time spent on licking the hindpaw. Both Fxyd6 cKO mice and control mice showed little or no responses to the injection of saline (t = 0.09386, df = 8, p = 0.9275) (Figure 5(a)). However, a significant reduction in the response time was observed in Fxyd6 cKO mice, compared to control mice (t = 2.745, df = 10, p = 0.0207) (Figure 5(a)). This result largely links TRPV1 channel with the thermal phenotypes found in Fxyd6 cKO mice. Then, we further studied the relationship between FXYD6 and TRPV1, including co-expression and interaction. We found that about 41.9% FXYD6-positive neurons expressed TRPV1, while about 19.8% TRPV1-positive neurons expressed FXYD6 (Figure 5(b)). Furthermore, Co-immunoprecipitation (Co-IP) results demonstrated that FXYD6 could interact with TRPV1 in the DRG (Figure 5(c)). Meanwhile, Co-IP showed in transfected HEK293T cells, FXYD6 also interacted with TRPV1 (Figure 5(d)). These data suggest that FXYD6 co-expresses and interacts with TRPV1 in C1 type of mechanoheat nociceptors.

**TRPV1 function is impaired in FXYD6-deficient, Gal+ small DRG neurons**

The DRG neurons were acutely disassociated and cultured, and small DRG neurons were patch-clamped with...
The currents of capsaicin-responsive DRG neurons from Fxyd6 cKO mice were decreased, compared with control mice ($t = 4.423$, df = 52, $p < 0.001$) (Figure 6(a)). After recording, we collected the DRG neurons, extracted total RNA from these neurons and obtained cDNA for single-cell PCR by reverse transcription. Then, we classified these recorded DRG neurons into different types according to the markers of different neuron types detected in these neurons (Figure 6(b)). In the Gal$^+$ C1 type of DRG neurons, but not Th$^+$ C3 type of DRG neurons, we found that the capsaicin-induced currents were decreased significantly in the Trpv1$^+$/Fxyd6$^-$ neurons, compared to Trpv1$^+$/Fxyd6$^+$ neurons ($t = 2.385$, df = 29, $p = 0.0238$) (Figure 6(c)). In addition, in HEK293 cell line, the heterologous expression of FXYD6 could also increase the TRPV1 capsaicin-sensitive currents ($t = 2.030$, df = 43, $p = 0.0486$) (Figure 6(d)). These results suggest that FXYD6 could upregulate the TRPV1 activity.

The PGDEE motif of FXYD6 is required for the FXYD6/TRPV1 interaction and FXYD6-mediated enhancement of TRPV1

Taken into the consideration that the mouse FXYD6 protein consisted of 95 amino acids, we tried to screen the key amino acids for the FXYD6/TRPV1 interaction. We aligned the FXYD6 sequences of different species, and focused on the two conserved motifs, namely highly
negatively charged PGDEE motif and highly conserved PFXYDY motif (Figure 7(a)). The mouse FXYD6 mutant plasmids were constructed, in which these motifs were replaced with alanine (Ala, A). Co-IP showed that when the PGDEE motif was replaced into neutral amino acid alanine (PGDEEmut), the interaction between the FXYD6 PGDEEmut and TRPV1 were significantly decreased ($t = 7.787$, $df = 8$, $p < 0.001$ versus the PGDEEmut and FXYD6 CDS, $n = 5$; $t = 1.666$, $df = 10$, $p = 0.1267$ versus the PFXYDYmut and FXYD6 CDS, $n = 6$; $t = 1.745$, $df = 6$, $p = 0.1317$ versus the (PF+PG)mut and FXYD6 CDS, $n = 4$) (Figure 7(b) and (c)). The PGDEE motif, which is completely conserved in all FXYD6 sequences
Figure 7. The PGDEE motif of FXYD6 is required for the FXYD6/TRPV1 interaction. (a) Alignment of FXYD6 protein sequences of different species indicated the PGDEE motif was completely conserved. (b) In the lysate of HEK 293T cells co-transfected with the plasmids expressing TRPV1-Flag and FXYD6-Myc or FXYD6mut-Myc, Co-IP showed that FXYD6-Myc or FXYD6mut-Myc is present in the proteins precipitated by Flag antibodies. The immunoblot signal of PGDEEmut-Myc was weaker than that of FXYD6 CDS-Myc in cells co-expressing TRPV1-Flag. The immunoblot shown are representatives of four such experiments. (c) Quantitation showed that the interaction between PGDEEmut-Myc and TRPV1-Flag was weaker than that between FXYD6 CDS-Myc and TRPV1-Flag (t = 7.787, df = 8, ****p < 0.0001, PGDEEmut versus FXYD6 CDS, n = 5; t = 1.666, df = 10, p = 0.1267, PFXYDYmut versus FXYD6 CDS, n = 6; t = 1.745, df = 6, p = 0.1317, (PF+PG)mut versus FXYD6 CDS, n = 4). (d) In HEK293 cells, TRPV1-mCherry was co-transfected with FXYD6 (TRPV1 + FXYD6) or PGDEEmut (TRPV1 + PGDEEmut). The TRPV1 capsaicin-sensitive currents in the PGDEEmut group were significantly decreased when compared to those in the FXYD6 group (n = 5 for both TRPV1 + FXYD6 cells and TRPV1 + PGDEEmut cells, *p < 0.05, unpaired t test). Data shown are mean ± SEM.
of different species, displays its important role in mediating the FXYD6/TRPV1 interaction.

Next, we further wondered that the PGDEE motif of FXYD6 is indispensable for the FXYD6-mediated enhancement of TRPV1 or not, here we still used the HEK293 cell line, and we co-transfected TRPV1-mCherry with FXYD6 (TRPV1+FXYD6) or PGDEEmut (TRPV1+PGDEEmut) in the HEK293 cell line. The TRPV1 capsaicin-sensitive currents in the PGDEEmut group were significantly decreased when compared to those in the FXYD6 group found ($t = 2.342$, df = 8, $p = 0.0473$) (Figure 7(d)). These results suggest that PGDEE motif is also required for the FXYD6-mediated enhancement of TRPV1.

**TAT-PGDEE elevates the threshold of response to the noxious thermal stimulation**

As FXYD6 could interact with TRPV1 channel via its PGDEE motif, we wondered whether the PGDEE motif was also involved in thermal nociception. Taking advantage of cell-penetrating peptide HIV-1 TAT (GRKKRRQRRRPQ), we designed the TAT-PGDEE, and the corresponding TAT-PGDEE scramble peptide (TAT-Scramble). Then the wildtype mice were intrathecally injected with the TAT peptides or saline. Hotplate test showed that TAT-PGDEE could elevate the thermal threshold in C57 mice, but TAT-Scramble or saline could not ($F_{1,128} = 28.36$, $p < 0.0001$ versus the TAT-PGDEE group and the TAT-Scramble group; $F_{1,112} = 20.83$, $p < 0.0001$ versus the TAT-PGDEE group and the Saline group; $n = 11$ for TAT-PGDEE group, $n = 7$ for the TAT-Scramble group and $n = 5$ for the Saline group) (Figure 8). Therefore, the PGDEE motif of FXYD6 is not only required for the FXYD6/TRPV1 interaction, but also plays an important role in thermal nociception.

**Discussion**

The present study showed that FXYD6 co-expresses with TRPV1 in Gal$^+$ C1 type of small DRG neurons. FXYD6 could interact with TRPV1 and increase the capsaicin-sensitive currents via TRPV1. In Fxyd6 cKO mice, TRPV1 capsaicin-sensitive currents were significantly decreased in Gal$^+$ C1 type of DRG neurons. Fxyd6 cKO mice showed impaired behavioral response to noxious heat. The highly negatively charged C-terminal PGDEE motif of FXYD6 was required for the FXYD6/TRPV1 interaction and FXYD6-mediated enhancement of TRPV1, and intrathecal TAT-PGDEE could elevate thermal threshold. These findings reveal that FXYD6 could promote the thermal nociception through regulating TRPV1 activity in a specific type of DRG neurons.

**FXYD6/TRPV1 interaction involves in thermal nociception**

FXYD6 is an unnecessary subunit of Na$^+$,K$^+$-ATPase, and could decrease the activity of Na$^+$,K$^+$-ATPase. Our previous studies demonstrate that secreted protein FSTL1 and membrane protein FXYD2 could regulate Na$^+$,K$^+$-ATPase activity distinctly, resulting in the alterations of neuronal excitability and threshold of somatosensation. Here, we found that besides the FSTL1/FXYD2/NKA system, the FXYD6/TRPV1 interaction also could play a role in somatosensation. In present study, we found that FXYD6 and TRPV1 co-expressed in Gal$^+$ mechanoheat nociceptor. The cKO of Fxyd6 impaired the behavioral responses to noxious heat and intraplantar injection of capsaicin. The defects of thermal nociception in Fxyd6 cKO mice were not mainly due to the loss of the regulatory effect of FXYD6 on Na$^+$, K$^+$-ATPase, because the responses to the noxious mechanical stimuli were not apparently altered in the mutant mice, and the physical blockage of the FXYD6/TRPV1 interaction could elevate the thermal threshold in wildtype of mice. Therefore, we provide an evidence that FXYD6 is involved in thermal nociception by interacting with TRPV1 channel in DRG neurons.

**FXYD6 increases the TRPV1 activity in the specific type of DRG neurons**

The single-cell RNA sequencing shows that Trpv1 is mainly expressed in Gal$^+$, Sst$^+$ (C2 type) and
Mrgrpa3+ (C4 type) DRG neurons that are also itch-sensing neurons, indicating that TRPV1 in different types of DRG neurons may have distinct functions. TRPV1-null mice and TRPV1-DTA mice lose the responses not only to heat, but also to pruritogenic chemical compounds. Actually, ablation of Stx+ DRG neurons or Mrgrpa3+ neurons reduces the scratching behavior, but not change the thermal threshold. Re-introducing TRPV1 in Mrgrpa3+ DRG neurons in TRPV1-null mice increases itch behavior, but little or no pain behavior. Our present study demonstrates that TRPV1-null mice increases itch behavior, but little or no change the thermal threshold.36,37

PGDEE motif is required for the FXYD6/TRPV1 interaction and thermal nociception

In the present study, we found that FXYD6 interacted with TRPV1 channel and promoted thermal nociception. FXYD6 worked as a regulator of TRPV1 to increase the capsaicin-induced currents. Different regulators of TRPV1 usually binds the TRPV1, and further change the initial current of TRPV1 or desensitization of TRPV1. Membrane protein Pirt binds to TRPV1 via its positively charged C terminus, and further enhance activity of TRPV1.27 SHANK3 interacts with TRPV1 via its proline-rich region, and regulates TRPV1.29 KChIP3 N-terminal 31–50 fragment binds to TRPV1, and decreases the surface localization of TRPV1.38 Phosphorylation status of Thr370 in TRPV1 dictates the association of β-arrestin 2 and TRPV1, and β-arrestin 2 mediates the desensitization of TRPV1.28 One study shows that calmodulin binds to the NH2-terminal ANK1 domain of TRPV1, while another study shows that calmodulin could bind to the C-terminal ANK1 of TRPV1 and mediates the desensitization of TRPV1.26,39 Our present study identified the PGDEE motif of FXYD6 as a key site required for the FXYD6/TRPV1 interaction. The PGDEE motif is highly negatively charged, and completely conserved in all FXYD6 protein sequences of different species, suggesting the importance of PGDEE motif. Therefore, we proposed that the FXYD6/TRPV1 interaction was mainly due to electrostatic interaction between the PGDEE motif of FXYD6 and the specific motif of TRPV1. Crystal structure of TRPV1 demonstrates that TRPV1 is a tetrameric protein and each monomer has six transmembrane segments.40,41 According to the structure of TRPV1 and FXYD1 (homologue of FXYD6), we speculated that the positively charged motif in ankyrin repeat domain of TRPV1 may participate in the FXYD6-TRPV1 interaction.42,43 Besides, we found that blocking the FXYD6/TRPV1 interaction could elevate the thermal threshold. Thus, the PGDEE motif of FXYD6 is not only required for the FXYD6/TRPV1 interaction, but also for the thermal nociception. Our study provides the evidence that FXYD6 promotes the thermal nociception through interacting with TRPV1 channel, suggesting its potential for pain therapy.

Author Contributions
X. Z., L. B., and K.-C. L. designed research; H. L., B. C., J. P. and H.-X. S. performed research; K.-K. W. provided single-cell RNAseq data; H. L. analyzed data; X. Z., L. B., K.-C. L. and H. L. wrote the paper.

Declaration of Conflicting Interests
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