Transmembrane channel-like (*tmc*) gene regulates *Drosophila* larval locomotion

Yanmeng Guo[a,b,c,d,e,f,1], Yuping Wang[a,b,1], Wei Zhang[c,d,e,f,1], Shan Meltzer[c,d,e,f,1], Damiano Zanini[g,1], Yue Yu[h], Jiefu Li[c,d,e,f], Tong Cheng[c,d,e,f], Zhenhao Guo[c,d,e,f], Qingxiu Wang[a], Julie S. Jacobs[i], Yashoda Sharma[i], Daniel F. Eberl[i], Martin C. Göpfert[g], Lily Yeh Jan[c,d,e,f], Yuh Nung Jan[c,d,e,f,2], and Zuoren Wang[a,2]

[a]Institute of Neuroscience, Chinese Academy of Sciences (CAS) Center for Excellence in Brain Science and Intelligence Technology, State Key Laboratory of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China; [b]Graduate School of University of Chinese Academy of Sciences, Beijing 100049, China; [c]Department of Physiology, University of California, San Francisco, CA 94158; [d]Department of Biochemistry, University of California, San Francisco, CA 94158; [e]Department of Biophysics, University of California, San Francisco, CA 94158; [f]Howard Hughes Medical Institute, University of California, San Francisco, CA 94158; [g]Department of Cellular Neurobiology, University of Göttingen, 37077 Goettingen, Germany; [h]College of Biological Sciences, China Agricultural University, Beijing 100193, China; and [i]Department of Biology, The University of Iowa, Iowa City, IA 52242

Contributed by Yuh Nung Jan, May 6, 2016 (sent for review February 24, 2016; reviewed by Wesley Grueber, Chun-Fang Wu, and Bing Ye)

Drosophila **larval locomotion, which entails rhythmic body contractions, is controlled by sensory feedback from proprioceptors. The molecular mechanisms mediating this feedback are little understood. By using genetic knock-in and immunostaining, we found that the *Drosophila melanogaster transmembrane channel-like* (*tmc*) gene is expressed in the larval class I and class II dendritic arborization (da) neurons and bipolar dendrite (bd) neurons, both of which are known to provide sensory feedback for larval locomotion. Larvae with knockdown or loss of *tmc* function displayed reduced crawling speeds, increased head cast frequencies, and enhanced backward locomotion. Expressing *Drosophila* TMC or mammalian TMC1 and/or TMC2 in the *tmc*-positive neurons rescued these mutant phenotypes. Bending of the larval body activated the *tmc*-positive neurons, and in *tmc* mutants this bending response was impaired. This implicates TMC's roles in *Drosophila* proprioception and the sensory control of larval locomotion. It also provides evidence for a functional conservation between *Drosophila* and mammalian TMCs.**

proprioception | locomotion | mechanosensation

Proprioception—the sense of positions, orientations, and movements of body parts—provides sensory feedback information for animals to maintain the right gestures and coordinate their body movements (1, 2). It has been known for centuries that proprioception is mediated by mechanosensitive proprioceptors (1, 3) such as mammalian muscle spindles and Golgi tendon organs (2, 3). In insects, mechanosensory campaniform sensilla, trichoform sensilla, chordotonal organs, and stretch receptors reportedly serve proprioceptive roles (4–10).

Proprioceptors integrate various mechanical cues, which are thought to be detected by mechanogated ion channels (11), to keep track of the relative positions and to coordinate the movements of different parts of the body (11). The molecular mechanisms underlying proprioceptive transduction have just begun to be elucidated. Among mechanosensitive ion channel candidates such as certain members of the transient receptor potential (TRP) channels (12–14), degenerin/epithelial sodium channels (DEG/ENaC) (15–17), Piezo (18–20), transmembrane channel-like (TMC) (21), K2P (22–24), and MscL (large-conductance mechanosensitive channel) (24, 25), TRPN and DEG/ENaC ion channels have been proposed to be mechanosensitive ion channels involved in proprioception in nematodes (26, 27), fruit flies (6, 9, 10) and zebrafish (28). Recently, it was reported that Piezo2 is essential for the mechanosensitivity of mammalian proprioceptors (29). However, whether other putative mechanosensitive ion channels participate in proprioception has remained unclear.

Tmc1, the founding member of the TMC gene family, was first reported for its role in auditory sensation owing to its genetic linkage to human deafness (30, 31) and its requirement for hearing in rodents (31). Eight *transmembrane channel-like* (*tmc*) genes have been identified in human and mouse genomes (32, 33). It has been suggested that TMC1 and TMC2 are likely essential components of the transduction channel complex in hair cells of the mouse inner ear (21, 34). The *tmc-1* gene in nematode was reported to encode a sodium-sensitive cation channel and participates in sensing high concentrations of sodium (35), suggestive of diverse functions of the *tmc* genes. There is only one *tmc* gene (CG46121) in the *Drosophila* genome (32, 33), providing an opportunity to study potentially diverse roles of the *tmc* gene.

Here we report a previously unidentified role of the *Drosophila tmc* gene. We found that *Drosophila* TMC is expressed and functions in larval class I and class II dendritic arborization (da) neurons and bipolar dendrite (bd) neurons and likely provides sensory feedback for larval locomotion (7, 9). Larvae with loss-of-function mutation of *tmc* exhibited defective locomotion, and this phenotype can be rescued by expressing *Drosophila* TMC or mouse TMC1 and/or TMC2 in *tmc*-positive neurons. Our study shows that *Drosophila* TMC contributes to mechanosensation in the body-wall sensory neurons and plays a role in locomotion of *Drosophila* larvae.

Significance

Locomotion requires peripheral sensory feedback from mechanosensitive proprioceptors. The molecular mechanisms underlying this proprioceptive locomotion control are largely unknown. Here we report that *tmc*, the *Drosophila* ortholog of the mammalian deafness gene *tmc1*, is expressed in larval peripheral sensory neurons and that these neurons require transmembrane channel-like (TMC) to respond to bending of the larval body. We further report that loss of TMC function causes locomotion defects. Finally, mammalian TMC1/2 are shown to rescue locomotion defects in *tmc* mutant larvae, providing evidence for a functional conservation between *Drosophila* and mammalian TMC proteins.

Author contributions: Y.G., Y.W., W.Z., S.M., D.F.E., M.C.G., Y.N.J., and Z.W. designed research; Y.G., Y.W., W.Z., S.M., D.Z., Y.Y., J.L., T.C., Z.G., Q.W., J.S.J., and Y.S. performed research; Y.G., Y.W., W.Z., S.M., D.Z., Y.Y., J.L., T.C., Z.G., Q.W., and J.S.J. contributed new reagents/analytic tools; Y.G., Y.W., W.Z., and S.M. analyzed data; and Y.G., Y.W., W.Z., S.M., D.Z., L.Y.J., Y.N.J., and Z.W. wrote the paper.

Reviewers: W.G., Columbia University; C.-F.W., University of Iowa; and B.Y., University of Michigan.

The authors declare no conflict of interest.

[1]Y.G., Y.W., W.Z., S.M., and D.Z. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: yuhnung.jan@ucsf.edu or zuorenwang@ion.ac.cn.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1606537113/-/DCSupplemental.

NEUROSCIENCE

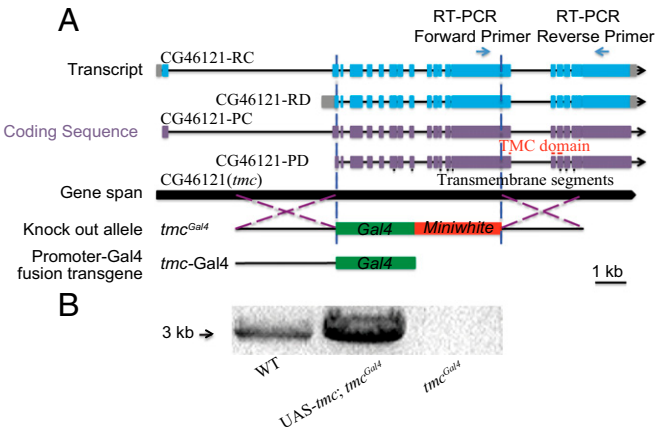

Fig. 1. Generation of *tmc* reporter and mutant alleles. (*A*) Targeting schemes for the generation of the tmc^{Gal4} mutant allele by homologous recombination and the generation of the *tmc*-Gal4 transgene. Black bars under CG46121-PD indicate the predicted transmembrane segments of *Drosophila* TMC (33). Red Bars above CG46121-PD indicate the predicted TMC domain (32). (*B*) Confirmation of the tmc^{Gal4} mutation as a null allele for transcript expression by RT-PCR.

Results

Generation of *Drosophila tmc* Mutants. The *tmc* genes encode proteins with putative transmembrane domains that potentially function as channels (32, 33). *Drosophila* has one *tmc* gene (CG46121), which is evolutionarily related to mammalian *tmc1* and *tmc2* genes (32, 33). The *Drosophila* TMC protein sequence is highly conserved with TMC family members in other species, particularly in the predicted transmembrane domains that contain the characteristic TMC motif (Fig. S1), although it has a much longer loop flanked by two putative transmembrane domains (Fig. S1).

To investigate the *Drosophila tmc* gene function, we first generated a transgenic reporter allele, *tmc*-Gal4, and one *tmc* knock-in reporter allele, tmc^{Gal4}, in which the bulk of the coding sequence is removed (Fig. 1*A*). For tmc^{Gal4}, the *Gal4* gene and the *miniwhite* gene were inserted into the *tmc* locus near the translation initiation codon of the *Drosophila* TMC isoform PD via ends-out homologous recombination (36, 37) so that about 7,000 bp of the *tmc* gene were replaced by the reporter construct (Fig. 1*A*). As a result, more than 1,000 amino acids, about one-half of the coding sequence of *tmc*, were deleted in the tmc^{Gal4} allele, likely resulting in a null mutation of the *tmc* gene. This was confirmed by RT-PCR, which detected no *tmc* RNA in tmc^{Gal4} larvae (Fig. 1*B*). *tmc* RNA was restored when we expressed a UAS-*tmc* rescue construct in tmc^{Gal4} mutants via the Gal4 (Fig. 1*B*).

***Drosophila* TMC Is Expressed in Class I da Neurons, Class II da Neurons, and bd Neurons.** To gain first insights into the roles of *Drosophila* TMC, we first tested whether *tmc* is expressed in sensory neurons in *Drosophila* larvae by using the Gal4/UAS system. The *tmc*-Gal4 and tmc^{Gal4} lines were used to drive the expression of the reporter UAS-mCD8-GFP. As revealed by the morphology and the position of the mCD8-GFP–positive neurons, class I and class II da neurons as well as bd neurons are labeled with both *tmc*-Gal4 and tmc^{Gal4} (Fig. 2 *A* and *B*). We have also generated another independent *tmc*-Gal4 line and observed expression in the same cells (Fig. S2). In adult flies, *tmc*-Gal4 labeled subsets of neurons in the mouth parts, olfactory neurons in the antenna (38), wing bristle neurons (39), haltere neurons (40), arista neurons (41), and many other sensory neurons (Fig. S2), including a subset of chordotonal (Cho) neurons (42), the hearing neurons of the fly (Fig. S2). This broad expression pattern of *tmc* raises the possibility that the single *Drosophila tmc* gene might have multiple functions in different sensory organs, which might be split among the eight mammalian members of the TMC family.

To examine the intrinsic expression pattern of *Drosophila* TMC, we generated an antibody against the C terminus of the *Drosophila* TMC protein. We first demonstrated the specificity of the antibody by immunostaining *Drosophila* TMC ectopically expressed in the fly brain neurons via Cha-Gal4 or in cultured *Drosophila* S2 cell lines. We found that immunofluorescence could be detected only in *Drosophila* TMC-expressing cells, suggesting that the antibody is specific (Fig. S3). Immunostaining of the larval body wall identified *Drosophila* TMC in class I and II da neurons and bd neurons in wild-type larvae (Fig. 2*A*), although no obvious immunofluorescence could be observed in *tmc* mutant larvae (Fig. 2*B*). Moreover, the immunofluorescence was restored by expressing of *Drosophila* TMC in *tmc* mutants (Fig. 2*C*). All of these results suggest that the antibody specifically recognizes *Drosophila* TMC proteins that are expressed in class I and class II da neurons and bd neurons.

TMC Regulates Larval Locomotion. Given that class I da neurons and bd neurons that express *tmc* (Fig. 2*A*) are proprioceptors that provide feedback on larval crawling (7, 9), we examined the locomotion behavior of *tmc* mutant larvae. The locomotion trajectories of tmc^{Gal4} larvae were distinct from those of wild-type w^{1118} larvae (Fig. 3*A*). We found that the crawling speeds of tmc^{Gal4} larvae were significantly reduced compared with control

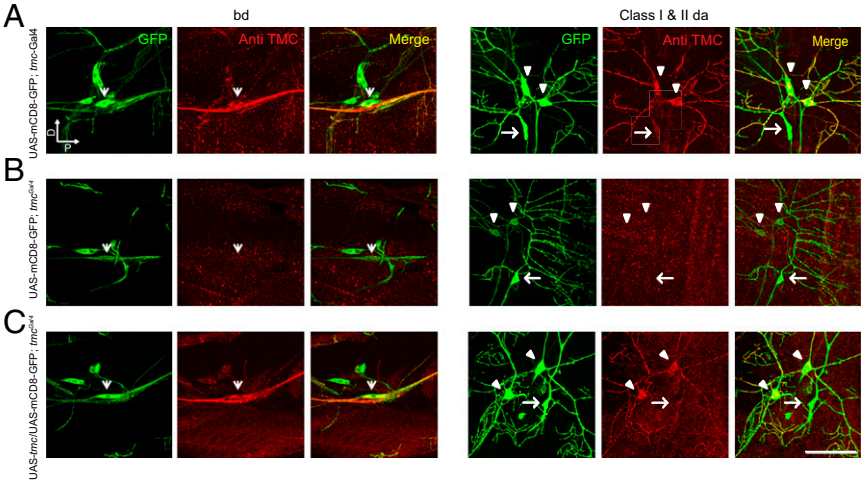

Fig. 2. Expression pattern of *tmc*. (*A*) Labeling via *tmc*-Gal4 and endogenous expression of *Drosophila* TMC in class I and class II da neurons and bd neurons. (*B*) Labeling via tmc^{Gal4} revealing the loss of expression of *Drosophila* TMC in class I and class II da neurons and bd neurons. (*C*) Restoration of *Drosophila* TMC expression in class I and class II da neurons and bd neurons by expressing *Drosophila* TMC. Arrowheads: bd neuron. Triangle: class I da neuron. Arrows: class II da neuron. Green indicates GFP signals, and red indicates TMC immunofluorescent signals. (*A*, *Left*) "D" indicates dorsal, and "P" indicates posterior. (Scale bar, 50 μm.)

larvae (Fig. 3*B*). Given that *Drosophila* larval locomotion includes several different types of movements, including linear forward crawling, turns, backward locomotion, and other movements (43, 44), we then analyzed the locomotion behavior in details. We found that tmc^{Gal4} larvae exhibited a significantly enhanced head curl behavior, with the head curling to the left, right, or up, whereas the abdomen remained still (Fig. 3*C* and Movies S1 and S2); the larvae also showed increased backward locomotion (Fig. 3*D* and Movies S1 and S2). Both these phenotypes characterize abnormal locomotion behaviors that ensue from the loss of proprioceptive feedback (7). To confirm that these behavioral defects are indeed caused by *tmc* mutation, we first crossed tmc^{Gal4} mutants with deficient flies that harbor a genomic deletion covering the *tmc* gene. We found that the behavioral defects of *tmc* mutants could not be complemented by the deletion allele (Fig. 3 *E* and *F*). Moreover, we found that knocking down the *tmc* gene by crossing *tmc*-Gal4 flies with UAS-*tmc*-RNAi flies led to similar although less severe behavioral defects, including enhanced head curl behavior and a tendency for backward movements (Fig. 3 *G* and *H*). Furthermore, we found that the behavioral defects in the tmc^{Gal4} larvae were fully rescued by expressing *Drosophila* TMC using the Gal4 driver in tmc^{Gal4} (Fig. 3 *A*–*D*). Taken together, our results demonstrate that *Drosophila* TMC is required for the normal crawling behavior in *Drosophila* larvae, likely functioning in class I and class II da neurons and bd neurons.

***Drosophila* TMC-Positive Neurons Show *Drosophila* TMC-Dependent Response to Body Bending.** Mutation of *Drosophila tmc* did not cause obvious defects in the dendrite morphology of class I da neurons or axon targeting of *tmc*-Gal4–positive neurons (Fig. S4), indicating that TMC protein might participate in mechanotransduction rather than neural development. Class I da neurons and bd neurons extend their dendrites along the anterior–posterior body axis, potentially facilitating their sensitivity to body contraction and relaxation during locomotion. To test if they could sense body-wall deformations in a manner that requires *tmc* gene function, we performed Ca^{2+} imaging of the axon terminals of the *tmc*-expressing neurons inside the ventral nerve cord (VNC) of larvae with or without the loss-of-function mutation tmc^{Gal4}. The Ca^{2+} level of these axon terminals was

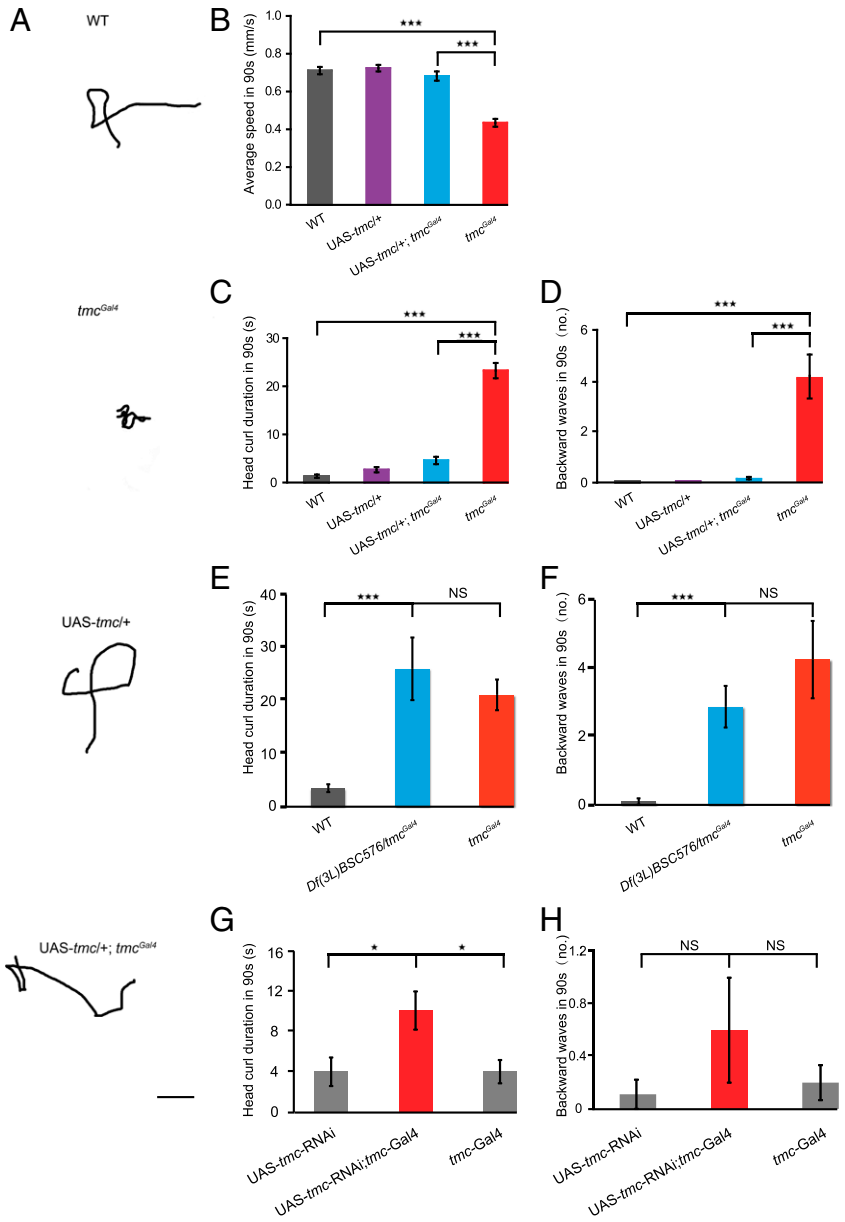

Fig. 3. *tmc* is important for *Drosophila* larval locomotion. (*A*) Crawling trajectories of wild-type w^{1118} larvae, tmc^{Gal4} mutants, UAS-*tmc* controls, and UAS-*tmc*; tmc^{Gal4} larvae. (Scale bar, 1 cm.) Restoring *tmc* expression rescued the locomotion defects in locomotion speed (*B*), head curl duration in 90 s (*C*), and backward wave numbers (*D*) in 90 s. One-way ANOVA followed by Tukey's HSD post hoc test was used to test for the statistical significance of the differences between wild-type w^{1118}, tmc^{Gal4}, UAS-*tmc*, and rescue (UAS-*tmc*; tmc^{Gal4}) larvae. ***$P < 0.001$. (*E*) Head curl duration (in 90 s) and (*F*) backward wave numbers (in 90 s) are comparable between tmc^{Gal4} and Df(3L)BSC576/tmc^{Gal4} larvae, with significant increase compared with wild-type control. One-way ANOVA was used to test for the statistical significance of the differences between tmc^{Gal4}, UAS-*tmc*, and rescue (UAS-*tmc*; tmc^{Gal4}) larvae, followed by Tukey's HSD post hoc test. ***$P < 0.001$. NS, not significant. (*G*) Head curl duration in 90 s and (*H*) backward wave numbers in 90 s of *tmc*-Gal4, UAS-*tmc*-RNAi, and *tmc* knockdown (UAS-*tmc*-RNAi; *tmc*-Gal4) larvae. One-way ANOVA was used to test for the statistical significance of the differences between *tmc*-Gal4, UAS-*tmc*-RNAi, and *tmc* knockdown (UAS-*tmc*-RNAi; *tmc*-Gal4) larvae, followed by Tukey's HSD post hoc test. *$P < 0.05$. NS, not significant. $n > 10$.

NEUROSCIENCE

monitored while the posterior portion of the body that contains the corresponding *tmc*-expressing cell bodies was bent to an angle of at least 45 degrees (Fig. 4*A*). In the control animals, *tmc*-Gal4–positive neurons were sensitive to body curvature, as the Ca^{2+} level was elevated by abdominal bending (Fig. 4 *B* and *C*). This response was significantly reduced in the *tmc* mutant larvae (Fig. 4 *B* and *C*). *Drosophila* TMC appears to play a role in the mechanotransduction of the class I da neurons and bd neurons that serve as proprioceptors during larval locomotion. However, heterologous expression of *Drosophila* TMC in S2 cells did not yield mechanosensitive channel activity (Fig. S5). Thus, it remains to be determined whether *Drosophila* TMC and/or other as-yet-unidentified channel proteins fulfill the function of mechanosensitive channels for proprioception in larval locomotion.

Functional Conservation Between *Drosophila* TMC and Mammalian TMC Proteins. As *Drosophila* TMC is the only fly homolog for mammalian TMC proteins (21, 34), we asked whether mammalian TMC proteins could functionally complement the *tmc* mutant defects in larval locomotion. To answer this question, we expressed mouse TMC1 and/or TMC2 in tmc^{Gal4}-labeled neurons of *tmc* mutant larvae and examined their locomotion behavior. Intriguingly, we found that expression of both mouse TMC1 and TMC2 could fully rescue the behavioral defects due to loss of *Drosophila tmc* function (Fig. 5 *A* and *B*). In light of the proposal that TMC1 and TMC2 might form heteromers to function as channels (21, 34), it is noteworthy that expressing either mouse TMC1 or TMC2 alone could also fully or partially rescue the behavioral defects (Fig. 5 *A* and *B*). Hence, mammalian TMC1 and TMC2 seem able to recapitulate the proprioceptive roles of *Drosophila* TMC.

Discussion

Larval Locomotion Pattern Is Regulated by *tmc* That Is Expressed in Class I and Class II da Neurons and bd Neurons. Proprioception is vital for animals to control their locomotion behavior, although the underlying mechanisms remain to be worked out in *Drosophila* and other animals (11). Here we report that the *tmc* gene contributes to proprioception and sensory feedback for normal forward crawling behavior in *Drosophila* larvae. We found that *tmc* is expressed in *Drosophila* larval sensory neurons (Fig. 2). Our behavioral and calcium imaging studies indicate that *Drosophila* TMC plays an important role in proprioception and regulation of crawling behavior (Figs. 3 and 4). Moreover, behavioral defects due to loss of *tmc* function in *Drosophila* were rescued by expressing mammalian TMC proteins, indicative of an evolutionarily conserved function (Fig. 5).

Differential Functions of Different Sensory Neurons in Regulating Locomotion. Several types of body-wall sensory neurons appear to play a role in the larval locomotion regulation. Silencing Cho neurons results in increased frequency and duration of turning and reduced duration of linear locomotion (5), a phenotype similar to that caused by *tmc* mutation, suggesting that the Cho neurons and the *tmc*-expressing neurons might converge to the same motor output pathway. Interestingly, blocking class IV da neurons produces an opposite phenotype—fewer turns (6). Given that the central projection of class IV da neurons in the VNC is distinct from that of class I da neurons and bd neurons (45), it will be interesting to see how they regulate the same behavior in opposing manners.

Different neurons might use different mechanosensitive ion channels in coordinating proprioceptive cues, similar to what has been found in the touch-sensitive neurons. The TRPN channel NOMPC functions in class III da neurons to mediate gentle touch sensation (13) whereas the DEG/ENaC ion channels PPK and PPK26, the TRP channel Painless, and Piezo function in class IV da neurons to mediate mechanical nociception (10, 20, 46–48). As to proprioception, chordotonal organs, class I and class IV da neurons and bd neurons may all contribute to proprioception to

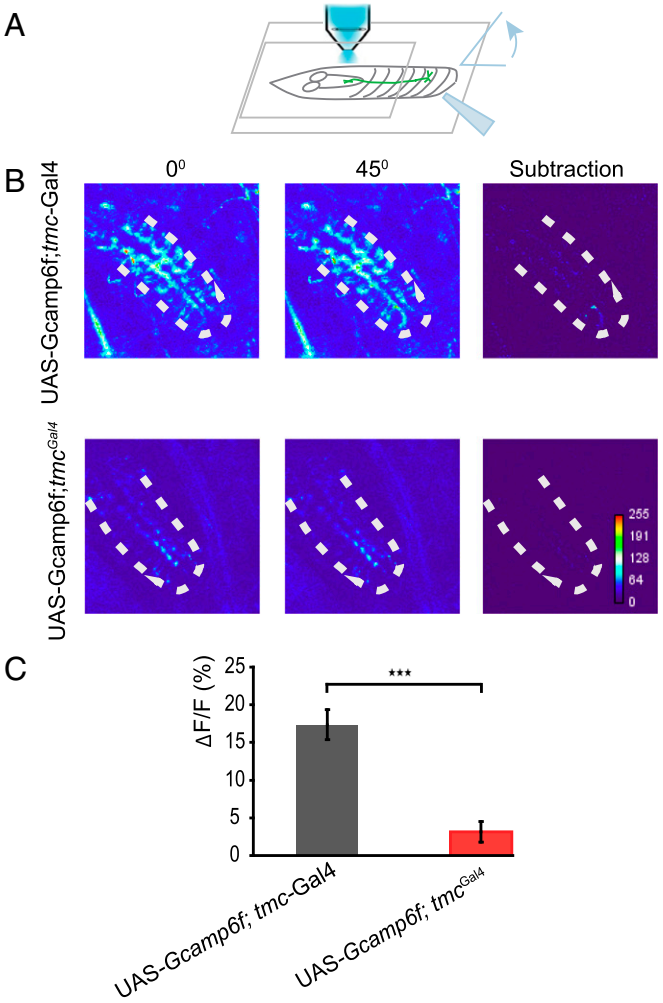

Fig. 4. Mechanosensitive calcium responses in the axon terminals of TMC-expressing neurons. (*A*) Experimental setup for imaging bending-evoked calcium signals in the axon terminals of TMC-expressing neurons. Bending was evoked using a glass probe. (*B*) Abdominal bending-evoked calcium signals in wild-type larvae and *tmc* mutant larvae. There is a GCaMP signal background difference between *tmc*-Gal4 and tmc^{Gal4} due to the different expression levels between them. (*C*) Statistical analysis of the calcium responses in wild-type and *tmc* mutant larvae. Two-tailed unpaired Student's *t* test was used to test the difference between wild-type w^{1118} and tmc^{Gal4}. ***$P < 0.001$. $n \geq 9$.

regulate larval locomotion behavior (5–8, 10). It is reported that NOMPC is expressed in class I da neurons and bd neurons, and mutations of NOMPC cause prolonged stride duration and reduced crawling speed of mutant larvae (9). In contrast, the DEG/ENaC ion channels PPK and PPK26 function in class IV da neurons to modulate the extent of linear locomotion; reduction of these channel functions leads to decreased turning frequency and enhanced directional crawling (6, 10).

Evolutionary Conservation of TMC Functions. *Drosophila* TMC protein exhibits sequence conservation with TMC family members in other species in the putative transmembrane domains, although it is much larger than its mouse or human homologs. It is of interest to determine whether the *Drosophila* TMC functions encompass a combination of functions of its mammalian homologs.

Among eight *tmc* genes in human and mice, *tmc1* and *tmc2* are found to be required for sound transduction in the hair cells of the inner ear (21, 30, 32–34, 49). However, these genes are very broadly expressed (30), so it is possible that they might also

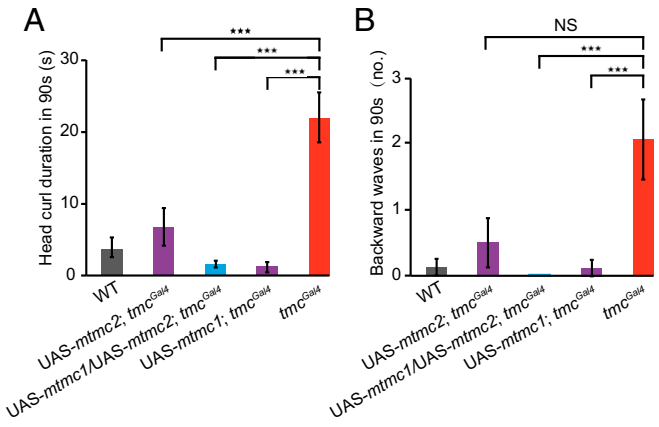

Fig. 5. Mammalian TMC1 and TMC2 rescue locomotion defects in *tmc* mutants. (*A*) Head curl duration (in 90 s) and (*B*) backward wave numbers (in 90 s) of wild-type, UAS-*mtmc2*; tmc^{Gal4}, UAS-*mtmc1*/UAS-*mtmc2*; tmc^{Gal4}, and UAS-*mtmc1*; tmc^{Gal4} larvae are significantly less than those of tmc^{Gal4} larvae. One-way ANOVA was used to test for the statistical significance of the differences, followed by Tukey's HSD post hoc test. ***$P < 0.001$. NS, not significant. $n \geq 8$.

function in other tissues. In light of our finding that *Drosophila* TMC functions in sensory neurons to regulate locomotion and mouse TMC1 or TMC2 functionally rescue the fly mutant phenotype, it will be interesting to test whether TMC1 and TMC2 have similar functions in addition to their involvement in hearing. Our work indicates that the *Drosophila tmc* gene participates in proprioception. Whether mammalian *tmc* genes, including *tmc1* and *tmc2*, participate in proprioception is an interesting open question.

In contrast to *tmc1* and *tmc2* in mammals and the *Drosophila tmc* gene, the *tmc-1* gene of *Caenorhabditis elegans* was reported to contribute to high sodium sensation in ASH polymodal avoidance neurons, in which TMC-1 ion channels could be activated by high concentrations of extracellular sodium salts and permeate cations (35). It will be of interest to explore the potential roles of *tmc* genes in various species in mechanosensation or osmosensation.

How mammalian TMC1 and TMC2 function in sound transduction is still not fully understood, and whether they are the pore-forming channel subunits is under debate (50, 51). It remains to be shown whether TMC1 and TMC2 can yield channel activities in heterologous expression systems (52, 53), and they likely require other proteins for their function in mechanotransduction (54–56). We have attempted to ectopically express the *Drosophila tmc* gene product in a variety of heterologous systems. However, no obvious mechanosensitive currents could be detected when these cells are exposed to mechanical stimuli (Fig. S5). One possibility is that the *Drosophila* TMC protein fails to be trafficked to plasma membrane in the expression system that we used. Alternatively, additional components are required to form a mechanosensitive complex as gating of certain mechanogated ion channels such as NOMPC (57) might require interactions of ion channels with extracellular matrix and/or intracellular cytoskeleton. Analyses of *Drosophila tmc* gene functions in larval locomotion regulation in this study, and in other future behavioral studies, may provide an opportunity to search for additional components that are necessary for the function of TMC proteins.

Materials and Methods

Fly Stocks and Genetics. Wild-type (w^{1118}) and UAS-*tmc*-RNAi flies (Vienna *Drosophila* Resource Center stock no. 42558) were used (58). For behavioral assays, flies were cultured in an incubator in 12-h dark/light cycles. Behavioral tests were performed blind to genotypes.

Molecular Cloning and Generation of Transgenic Flies. UAS-*tmc-attB* was generated by amplifying the *tmc*-coding sequence via RT-PCR and inserting the coding sequence into the pUAST-attB vector. P{nos-phiC31\int.NLS}X and P{CaryP}attP40 flies were used as the hosts for the transgenic insertion on the second chromosome. For UAS flies carrying mouse *tmc* genes, P{nos-phiC31\int.NLS}X, P{CaryP}attP40, and M{vas-int.Dm}ZH-2A, PBac{y[+]-attP-9A}VK00005 flies were used as the hosts for the transgenic insertion on the second and third chromosomes, respectively. UAS-*mtmc1* and UAS-*mtmc2* fly insertions were confirmed with PCR that detects a fraction of the *tmc* CDS. The *tmc1* and *tmc2* clones are a gift from Andrew Griffith, National Institute on Deafness and Other Communication Disorder, Bethesda. The *tmc*-Gal4 construct was generated by amplifying the *Drosophila tmc* promoter region via PCR (forward primer: ACGGTGGAATCCTGTTTGGTGA; reverse primer: CCTGCCTCGCTGTCCTTTGTAGA) and then cloning into the BamHI site of the pCasper-Aug-Gal4 vector. Transgenic flies were generated by *P*-element–mediated germ-line transformations.

Mutagenesis. The tmc^{Gal4} mutant fly was generated by ends-out homologous recombination (37). The 5′ and 3′ homologous arms of *tmc* were amplified from w^{1118} flies by PCR cloning and cloned into pw35-Gal4 vectors using the pEASY-Uni Seamless Cloning and Assembly Kit (Beijing TransGen Biotech Co.). Mutagenesis was performed as previously described (37, 48).

Antibodies and Immunostaining.

Antibody generation. The peptide containing the last 23 amino acids (1909–1932: CDPRSASPEPTVNIIRIDIENEHEK) was injected into rabbit for antibody generation (YenZym antibody). The antiserum was affinity-purified to obtain the antibody for *Drosophila* TMC protein.

S2 cell staining. S2 cells were fixed in 4% (wt/vol) PFA for 30 min at 4 °C. Cells were blocked with 10% (vol/vol) normal goat serum for 30 min at room temperature and then incubated with primary antibody [rabbit anti-TMC (1:200; YenZym Antibodies)] for 2 h and secondary antibody (Alexa 555-conjugated goat anti-mouse IgG 1:200; Invitrogen) for 1 h. After washing briefly, cells were mounted on coverslip for imaging.

Fly brain whole-mount staining. The whole brains were dissected out from Cha-Gal4; UAS-*tmc*-GFP flies and fixed in 4% (wt/vol) PFA for 30 min at 4 °C. The brains were blocked with 10% (vol/vol) normal goat serum for 30 min at room temperature and then incubated with primary antibody [rabbit anti-TMC (1:200; YenZym Antibodies)] overnight at 4 °C and with secondary antibody (Alexa 555-conjugated goat anti-mouse IgG 1:200; Invitrogen) for 2 h at room temperature. After washing briefly, brains were mounted on a coverslip for imaging.

Larval neuron staining. Larval body-wall neuron immunohistochemical staining was performed as reported previously (59), except that the mounting medium used was VectaShield (Vector Laboratories). The filleted larvae were fixed for 20 min at room temperature (RT) and blocked with blocking buffer for 1 h RT. The samples were then incubated in blocking buffer containing *Drosophila* TMC antibody (1:300) for 2 h RT and after washing with secondary antibody for 2 h RT. Slides were imaged on a Leica SP5 confocal microscope using an oil immersion 40× objective.

Behavioral Assays. The locomotion assay was performed similarly as previously described (5, 10). Videotaped locomotion behavior was analyzed offline using the Noldus software. Dislike turning behavior and head curl behavior duration were counted when the head of the larvae curled to the left, right, or up and its abdomen remained still. Backward locomotion numbers were counted when there was at least one backward wave of the whole body.

Calcium Imaging. A wandering third instar larva was picked up and rinsed with water. The larva was then mounted on a glass slide with the ventral side up. A glass slip was pressed on the anterior part of the larval body to reduce movement, and only the posterior segments were exposed to mechanical stimulation. A glass probe was used to push the larval body laterally to achieve a certain degree. Once the larval body achieved the certain degree, the glass probe was released. The imaging data were acquired in a Zeiss LSM510 confocal microscope. The newly available genetically coded calcium indicator GCaMP6f was used to measure the calcium signal. GCaMP6f was excited by 488-nm laser, and the fluorescent signals were collected as projections at a frame rate of about 8 Hz. The calcium signal was continuously collected before, during, and after the bending stimulation. The average GCaMP6f signal from the first 3 s before stimulus was taken as F_0, and $\Delta F/F_0$ was calculated for each data point.

S2 Cell Transfection and Electrophysiological Recording. S2 cell transfection, electrophysiological recording, and mechanical stimulation were performed as previously described (13). Briefly, *Drosophila* S2 cells were cultured at 25 °C in Schneider's medium with 10% (vol/vol) FBS. S2 cells were transfected with

an Effectene kit (Qiagen) in accordance with the product protocol. pUAST-*tmc*-GFP was cotransfected with pActin-Gal4. Electrophysiological recording was carried out 24–48 h after transfection. The bath solution contained 10 mM Hepes and 140 mM sodium methanesulfonate or 140 mM potassium methanesulfonate. The pipette solution contained 10 mM Hepes and 140 mM potassium Gluconic acid/140 mM cesium methanesulfonate. A glass probe or the recording pipette was used to give a mechanical stimulation or a negative/positive pressure, respectively. Movement steps of the glass probe were triggered and controlled by a Piezo amplifier or a Sutter MP285 manipulator. Pressure steps with a 10 mm·Hg increment were applied via a High Speed Pressure Clamp (HSPC, ALA-scientific), which was controlled and triggered by the pClamp software and Master-8.

ACKNOWLEDGMENTS. We thank Dr. Andrew Griffith (National Institute on Deafness and Other Communication Disorder) for mouse *tmc* clones; Dr. Craig Montell (University of California, Santa Barbara) for the pw35-Gal4 plasmids; the Vienna *Drosophila* Resource Center for UAS-*tmc*-RNAi fly stock; Susan Younger, Sandra Barbel, and Margret Winkler for technical support; Ulrich Müller and Bo Zhao (The Scripps Research Institute) for suggestions; and members of the Z.W. laboratory, L.Y.J. laboratory, D.F.E. laboratory, and M.C.G. laboratory for discussion. This work was supported by DFG Grants SFB 889 A1 and GO 1092/1-2 (to M.C.G.); a BRAINseed award (to Y.N.J.); NIH Grants R37NS040929 and 5R01MH084234 (to Y.N.J.) and DC004848 (to D.F.E.); the Strategic Priority Research Program (B) of the Chinese Academy of Sciences Grant XDB02010005 (to Z.W.); and China 973 Project Grant 2011CBA0040 (to Z.W.). L.Y.J. and Y.N.J. are investigators of the Howard Hughes Medical Institute.

1. Hasan Z (1992) Role of proprioceptors in neural control. *Curr Opin Neurobiol* 2(6): 824–829.
2. Dietz V (2002) Proprioception and locomotor disorders. *Nat Rev Neurosci* 3(10): 781–790.
3. Proske U, Gandevia SC (2012) The proprioceptive senses: Their roles in signaling body shape, body position and movement, and muscle force. *Physiol Rev* 92(4):1651–1697.
4. Kernan MJ (2007) Mechanotransduction and auditory transduction in *Drosophila*. *Pflugers Arch* 454(5):703–720.
5. Caldwell JC, Miller MM, Wing S, Soll DR, Eberl DF (2003) Dynamic analysis of larval locomotion in *Drosophila* chordotonal organ mutants. *Proc Natl Acad Sci USA* 100(26): 16053–16058.
6. Ainsley JA, et al. (2003) Enhanced locomotion caused by loss of the *Drosophila* DEG/ENaC protein Pickpocket1. *Curr Biol* 13(17):1557–1563.
7. Hughes CL, Thomas JB (2007) A sensory feedback circuit coordinates muscle activity in *Drosophila*. *Mol Cell Neurosci* 35(2):383–396.
8. Song W, Onishi M, Jan LY, Jan YN (2007) Peripheral multidendritic sensory neurons are necessary for rhythmic locomotion behavior in *Drosophila* larvae. *Proc Natl Acad Sci USA* 104(12):5199–5204.
9. Cheng LE, Song W, Looger LL, Jan LY, Jan YN (2010) The role of the TRP channel NompC in *Drosophila* larval and adult locomotion. *Neuron* 67(3):373–380.
10. Gorczyca DA, et al. (2014) Identification of Ppk26, a DEG/ENaC channel functioning with Ppk1 in a mutually dependent manner to guide locomotion behavior in *Drosophila*. *Cell Reports* 9(4):1446–1458.
11. Delmas P, Hao J, Rodat-Despoix L (2011) Molecular mechanisms of mechanotransduction in mammalian sensory neurons. *Nat Rev Neurosci* 12(3):139–153.
12. Kang L, Gao J, Schafer WR, Xie Z, Xu XZS (2010) *C. elegans* TRP family protein TRP-4 is a pore-forming subunit of a native mechanotransduction channel. *Neuron* 67(3):381–391.
13. Yan Z, et al. (2013) *Drosophila* NOMPC is a mechanotransduction channel subunit for gentle-touch sensation. *Nature* 493(7431):221–225.
14. Gong J, Wang Q, Wang Z (2013) NOMPC is likely a key component of *Drosophila* mechanotransduction channels. *Eur J Neurosci* 38(1):2057–2064.
15. O'Hagan R, Chalfie M, Goodman MB (2005) The MEC-4 DEG/ENaC channel of *Caenorhabditis elegans* touch receptor neurons transduces mechanical signals. *Nat Neurosci* 8(1):43–50.
16. Chatzigeorgiou M, et al. (2010) Specific roles for DEG/ENaC and TRP channels in touch and thermosensation in *C. elegans* nociceptors. *Nat Neurosci* 13(7):861–868.
17. Geffeney SL, et al. (2011) DEG/ENaC but not TRP channels are the major mechanoelectrical transduction channels in a *C. elegans* nociceptor. *Neuron* 71(5):845–857.
18. Coste B, et al. (2010) Piezo1 and Piezo2 are essential components of distinct mechanically activated cation channels. *Science* 330(6000):55–60.
19. Coste B, et al. (2012) Piezo proteins are pore-forming subunits of mechanically activated channels. *Nature* 483(7388):176–181.
20. Kim SE, Coste B, Chadha A, Cook B, Patapoutian A (2012) The role of *Drosophila* Piezo in mechanical nociception. *Nature* 483(7388):209–212.
21. Pan B, et al. (2013) TMC1 and TMC2 are components of the mechanotransduction channel in hair cells of the mammalian inner ear. *Neuron* 79(3):504–515.
22. Honoré E (2007) The neuronal background K2P channels: Focus on TREK1. *Nat Rev Neurosci* 8(4):251–261.
23. Alloui A, et al. (2006) TREK-1, a K+ channel involved in polymodal pain perception. *EMBO J* 25(11):2368–2376.
24. Nilius B, Honoré E (2012) Sensing pressure with ion channels. *Trends Neurosci* 35(8): 477–486.
25. Sukharev SI, Blount P, Martinac B, Blattner FR, Kung C (1994) A large-conductance mechanosensitive channel in *E. coli* encoded by mscL alone. *Nature* 368(6468): 265–268.
26. Tavernarakis N, Shreffler W, Wang S, Driscoll M (1997) *unc-8*, a DEG/ENaC family member, encodes a subunit of a candidate mechanically gated channel that modulates *C. elegans* locomotion. *Neuron* 18(1):107–119.
27. Li W, Feng Z, Sternberg PW, Xu XZS (2006) A *C. elegans* stretch receptor neuron revealed by a mechanosensitive TRP channel homologue. *Nature* 440(7084):684–687.
28. Sidi S, Friedrich RW, Nicolson T (2003) NompC TRP channel required for vertebrate sensory hair cell mechanotransduction. *Science* 301(5629):96–99.
29. Woo SH, et al. (2015) Piezo2 is the principal mechanotransduction channel for proprioception. *Nat Neurosci* 18(12):1756–1762.
30. Kurima K, et al. (2002) Dominant and recessive deafness caused by mutations of a novel gene, TMC1, required for cochlear hair-cell function. *Nat Genet* 30(3):277–284.
31. Keats BJ, et al. (1995) The deafness locus (dn) maps to mouse chromosome 19. *Mamm Genome* 6(1):8–10.
32. Kurima K, Yang Y, Sorber K, Griffith AJ (2003) Characterization of the transmembrane channel-like (TMC) gene family: Functional clues from hearing loss and epidermodysplasia verruciformis. *Genomics* 82(3):300–308.
33. Keresztes G, Mutai H, Heller S (2003) TMC and EVER genes belong to a larger novel family, the TMC gene family encoding transmembrane proteins. *BMC Genomics* 4(1):24.
34. Kawashima Y, et al. (2011) Mechanotransduction in mouse inner ear hair cells requires transmembrane channel-like genes. *J Clin Invest* 121(12):4796–4809.
35. Chatzigeorgiou M, Bang S, Hwang SW, Schafer WR (2013) *tmc-1* encodes a sodium-sensitive channel required for salt chemosensation in *C. elegans*. *Nature* 494(7435): 95–99.
36. Gong WJ, Golic KG (2003) Ends-out, or replacement, gene targeting in *Drosophila*. *Proc Natl Acad Sci USA* 100(5):2556–2561.
37. Moon SJ, Lee Y, Jiao Y, Montell C (2009) A *Drosophila* gustatory receptor essential for aversive taste and inhibiting male-to-male courtship. *Curr Biol* 19(19):1623–1627.
38. Vosshall LB (2000) Olfaction in *Drosophila*. *Curr Opin Neurobiol* 10(4):498–503.
39. Palka J, Malone MA, Ellison RL, Wigston DJ (1986) Central projections of identified *Drosophila* sensory neurons in relation to their time of development. *J Neurosci* 6(6): 1822–1830.
40. Cole ES, Palka J (1982) The pattern of campaniform sensilla on the wing and haltere of *Drosophila melanogaster* and several of its homeotic mutants. *J Embryol Exp Morphol* 71(Oct):41–61.
41. Foelix RF, Stocker RF, Steinbrecht RA (1989) Fine structure of a sensory organ in the arista of *Drosophila melanogaster* and some other dipterans. *Cell Tissue Res* 258(2): 277–287.
42. Jarman AP (2002) Studies of mechanosensation using the fly. *Hum Mol Genet* 11(10): 1215–1218.
43. Green CH, Burnet B, Connolly KJ (1983) Organization and patterns of inter specific and intraspecific variation in the behavior of *Drosophila* larvae. *Anim Behav* 31(Feb): 282–291.
44. Wang JW, et al. (1997) Morphometric description of the wandering behavior in *Drosophila* larvae: Aberrant locomotion in Na+ and K+ channel mutants revealed by computer-assisted motion analysis. *J Neurogenet* 11(3-4):231–254.
45. Grueber WB, et al. (2007) Projections of *Drosophila* multidendritic neurons in the central nervous system: Links with peripheral dendrite morphology. *Development* 134(1):55–64.
46. Tracey WD, Jr, Wilson RI, Laurent G, Benzer S (2003) *painless*, a *Drosophila* gene essential for nociception. *Cell* 113(2):261–273.
47. Zhong L, Hwang RY, Tracey WD (2010) Pickpocket is a DEG/ENaC protein required for mechanical nociception in *Drosophila* larvae. *Curr Biol* 20(5):429–434.
48. Guo Y, Wang Y, Wang Q, Wang Z (2014) The role of PPK26 in *Drosophila* larval mechanical nociception. *Cell Reports* 9(4):1183–1190.
49. Vreugde S, et al. (2002) Beethoven, a mouse model for dominant, progressive hearing loss DFNA36. *Nat Genet* 30(3):257–258.
50. Kim KX, et al. (2013) The role of transmembrane channel-like proteins in the operation of hair cell mechanotransducer channels. *J Gen Physiol* 142(5):493–505.
51. Beurg M, Kim KX, Fettiplace R (2014) Conductance and block of hair-cell mechanotransducer channels in transmembrane channel-like protein mutants. *J Gen Physiol* 144(1):55–69.
52. Holt JR, Pan B, Koussa MA, Asai Y (2014) TMC function in hair cell transduction. *Hear Res* 311:17–24.
53. Kawashima Y, Kurima K, Pan B, Griffith AJ, Holt JR (2015) Transmembrane channel-like (TMC) genes are required for auditory and vestibular mechanosensation. *Pflugers Arch* 467(1):85–94.
54. Xiong W, et al. (2012) TMHS is an integral component of the mechanotransduction machinery of cochlear hair cells. *Cell* 151(6):1283–1295.
55. Zhao B, et al. (2014) TMIE is an essential component of the mechanotransduction machinery of cochlear hair cells. *Neuron* 84(5):954–967.
56. Maeda R, et al. (2014) Tip-link protein protocadherin 15 interacts with transmembrane channel-like proteins TMC1 and TMC2. *Proc Natl Acad Sci USA* 111(35): 12907–12912.
57. Zhang W, et al. (2015) Ankyrin repeats convey force to gate the NOMPC mechanotransduction channel. *Cell* 162(6):1391–1403.
58. Dietzl G, et al. (2007) A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. *Nature* 448(7150):151–156.
59. Grueber WB, Jan LY, Jan YN (2002) Tiling of the *Drosophila* epidermis by multidendritic sensory neurons. *Development* 129(12):2867–2878.