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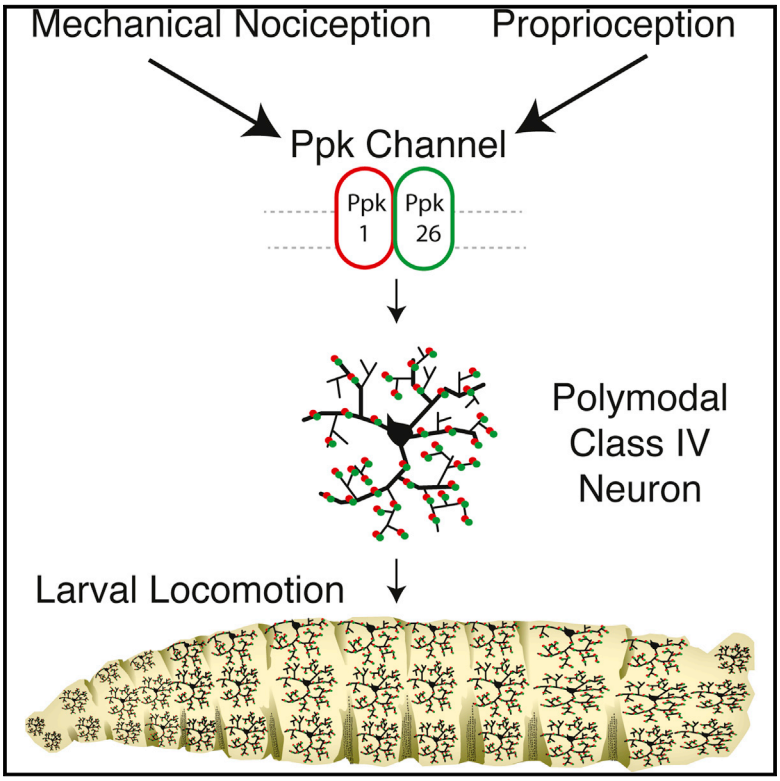
Graphical Abstract



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In Brief

Using a screen for ion channels in polymodal nociceptive neurons, Gorczyca et al. identify Ppk26, a member of the trimeric DEG/ENaC channel family, as necessary for both proper locomotive behavior and mechanical, but not thermal, nociception.

Highlights

Ppk26 is identified as a Ppk1-interacting DEG/ENaC channel subunit

Ppk26 and Ppk1 show mutual dependence for surface expression in dendrites

Ppk26 and Ppk1, but not Piezo, are critical for proper locomotion behavior

Ppk26 and Ppk1 are critical for mechanical, but not thermal, nociception behavior

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Identification of Ppk26, a DEG/ENaC Channel Functioning with Ppk1 in a Mutually Dependent Manner to Guide Locomotion Behavior in *Drosophila*

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SUMMARY

A major gap in our understanding of sensation is how a single sensory neuron can differentially respond to a multitude of different stimuli (polymodality), such as propio- or nocisensation. The prevailing hypothesis is that different stimuli are transduced through ion channels with diverse properties and subunit composition. In a screen for ion channel genes expressed in polymodal nociceptive neurons, we identified Ppk26, a member of the trimeric degenerin/epithelial sodium channel (DEG/ENaC) family, as being necessary for proper locomotion behavior in *Drosophila* larvae in a mutually dependent fashion with coexpressed Ppk1, another member of the same family. Mutants lacking Ppk1 and Ppk26 were defective in mechanical, but not thermal, nociception behavior. Mutants of Piezo, a channel involved in mechanical nociception in the same neurons, did not show a defect in locomotion, suggesting distinct molecular machinery for mediating locomotor feedback and mechanical nociception.

INTRODUCTION

The somatosensory system serves to integrate multiple modalities, including temperature sensation, mechanical cues, body posture (proprioception), and pain (nociception) (Lumpkin and Caterina, 2007). Stimuli can be either unimodal, responding to only one type of stimulus, or polymodal, responding to multiple stimuli. Nociceptors in the skin are an important class of somatosensory neurons either unimodally or polymodally dedicated to painful sensation of high threshold mechanical stimuli, noxious temperatures, chemical insult, or tissue damage. Mammalian nociceptors typically have multiple bare sensory dendrites with a highly elaborate dendritic pattern, with sensory receptors for each modality thought to reside in dendrites. Nociceptors of similar morphology and function are present in *Drosophila*, providing evidence for evolutionary conservation of this type of sensory neurons (Tracey et al., 2003).

Proprioceptors are another important class of sensory neurons involved in sensing body position during movement. Although poorly understood, peripheral proprioceptive mechanosensory input provides ongoing information on body position, necessary for tuning central mechanisms driving locomotion (Proske and Gandevia, 2012). Sensory ion channels, primary candidates mediating this process (Corey, 2006), reside in dendrites of multidendritic and ciliated sensory neurons (Desai et al., 2014).

Although some sensory neurons function only as proprioceptors, there is growing evidence that polymodal nociceptive neurons can also convey information about body position. Patients with congenital insensitivity to pain, arising from deficits in Aδ and C fiber function, but not from myelinated proprioceptive fibers, also have impaired proprioception (Axelrod and Hilz, 2003; Iijima and Haga, 2010; Rosemberg et al., 1994). "Sleeping" nociceptors in humans do not respond to painful stimuli unless sensitized by chronic injury (Ørstavik et al., 2003), and these could contribute proprioceptive or kinesthetic information in non-sensitized conditions. In *Caenorhabditis elegans*, PVD and FLP neurons are high-threshold mechanosensors mediating mechanical nociceptive behaviors but are also acting to coordinate body posture, locomotion, and temperature sensing (Way and Chalfie, 1989; Chatzigeorgiou et al., 2010; Arnadóttir et al., 2011; Liu et al., 2012). An open question is how a single neuron can distinguish between mechanosensory modalities, for example, those involved in proprioception and nociception.

The dendritic arborization (da) neurons in *Drosophila* larvae constitute a useful genetic system for the study of mechanisms underlying dendrite development and function via ion channels. da neurons are a set of four classes of well-defined segmentally repeated sensory neurons. Each class has dendritic arbors with a unique stereotypic pattern of arborization, presumably reflecting its functional requirement (Bodmer and Jan, 1987; Grueber et al., 2002). Of these, class III and class IV da neurons have dendrites that tile the epidermis.

Class III da neurons have actin-rich spine-like filopodia and are sensitive to gentle touch. These neurons express the TRPN channel NompC—localized to dendrites—and are involved in

mechanosensation of gentle touch (Tsubouchi et al., 2012; Yan et al., 2013; Kernan et al., 1994). Class I da and bd neurons also express NompC and are important for coordinating the appropriate timing of peristaltic locomotion; loss of NompC function in these neurons results in profound slowing and paralysis (Cheng et al., 2010).

Class IV da neurons express the degenerin/epithelial sodium channel (DEG/ENaC) Pickpocket (Ppk1) and play an essential role in coordinating turning behavior (Adams et al., 1998; Ainsley et al., 2003). The DEG/ENaC or Ppk channel superfamily are voltage insensitive and are assembled as either homomeric or heteromeric trimers. Interestingly, Ppk1 also plays an essential role in mechanical nociception behavior (Zhong et al., 2010), suggesting that these neurons can process multiple stimulus modalities. Class IV da neurons resemble mammalian nociceptors morphologically (Caterina and Julius, 1999; Tracey et al., 2003) and in their polymodal sensitivity to a variety of sensory stimuli (Ohyama et al., 2013). In addition to sensing mechanical nociception and coordinating locomotion, class IV da neurons are also sensitive to temperature (Hwang et al., 2012), light (Xiang et al., 2010), and chemical stimuli (Kang et al., 2010; Xiang et al., 2010). Recent studies show that the newly discovered mechanosensory ion channel, Piezo (Coste et al., 2010), functions in mechanical nociception in class IV da neurons (Kim et al., 2012) in a pathway that is parallel to Ppk1 with respect to mechanical nociception. How Ppk1 functions in parallel to Piezo during this process, is still unclear.

DEG/ENaC-type channels are often coexpressed in various parts of the nervous system. Of these, Acid-Sensing Ion Channels (ASICs) are gated by protons and are targets of potent analgesics (Diochot et al., 2012). Although gated by protons, this stimulus activates only a small fraction of the maximal ASIC conductance, and mounting evidence suggests the existence of more potent stimuli in vivo, acting independently or coincidently to protons (Bagriantsev and Minor, 2010; Bohlen et al., 2011; Yu et al., 2010). During tissue injury and acidosis, ASIC channels function in behavior and physiology directly related to pH change (Ziemann et al., 2009). However, other stimuli may regulate the channels at physiologic pH (Wemmie et al., 2013). In heterologous systems, differential ASIC subunit composition can result in distinct response properties, but heteromutimerization might also affect surface trafficking of these channels in vivo (Sherwood et al., 2011). In *C. elegans*, many MEC channels involved in mechanotransduction are coexpressed, such as the DEG/ENaC pore-forming subunits mec-4 and mec-10. These subunits, along with deg-1 act as mechanosensory ion channels for touch sensation in vivo (Geffeney et al., 2011; O'Hagan et al., 2005). However, loss of mec-10 does not result in complete loss of touch sensitivity but rather results in an atypical mechanoreceptor current (Arnadóttir et al., 2011).

In *Drosophila*, our knowledge about DEG/ENaC hetero-oligomerization is limited to coexpression of Ppk23 and Ppk29 in contact chemoreceptors (Thistle et al., 2012), Ppk11 and Ppk16 in motor neurons (Younger et al., 2013), and Ppk11 and Ppk19 in taste bristles (Liu et al., 2003). Ppk23 and Ppk29 play nonredundant and essential roles in contact chemoreceptors, with each mutant showing loss of calcium response to cuticular hydrocarbons and defective courtship. Ppk11 and Ppk16 are coexpressed in motor neurons as a single transcript in an operon-like fashion, and mutations in each or both subunits result in the same defect in homeostatic plasticity (Younger et al., 2013). Disrupting Ppk11 and Ppk19 also affects the ability to taste salt (Liu et al., 2003). Functional dependency on heteromeric channels could explain the nonredundancies observed in these cases, a possibility that remains to be tested.

Here, we report that the previously uncharacterized DEG/ENaC channel Ppk26/CG8546 is specifically expressed in class IV da neurons, which have been implicated in both mechanical nociception and proprioception. We find that Ppk26 and Ppk1 proteins colocalize in dendrites of class IV da neurons and that they interact biochemically. Analysis of specific Ppk1 and Ppk26 null mutants reveals a reciprocal dependence for plasma membrane localization in dendrites. Behavioral analysis of mutants and knockdowns suggest that Ppk26 and Ppk1 are likely to function together during locomotor behavior, presumably through the transduction or processing of a proprioceptive cue. We also report that both Ppk1 and Ppk26 play a role in mechanical, but not thermal, nociception behavior. Surprisingly, we found that Piezo null mutants have normal locomotion, suggesting that whereas Piezo might function in parallel with Ppk1 in mechanical nociception, they participate in a separate pathway during locomotion. The identification of Ppk26 as a DEG/ENaC subunit that is coexpressed with Ppk1, possibly in the same channel complex, as well as the reciprocal requirement for their dendritic plasma membrane localization, provides insights into the function of DEG/ENaC channels during mechanosensitive behaviors.

RESULTS

Ppk26 Is a Member of the DEG/ENaC Family

Sensory modality is thought to be in large part determined by the complement of sensory ion channels expressed in a neuron. In a screen for ion channels specifically expressed in larval class IV da neurons, we identified Ppk26 as a strong candidate. By fusing a 0.8 kB enhancer sequence upstream of the *ppk26* transcriptional start sequence to a minimal promoter and the Gal4 element, we generated the *ppk26-Gal4* driver, which was found to express Gal4 exclusively in class IV da neurons (Figures 1A and 1B). A similar enhancer of 2.2 kB upstream of Ppk26 was independently observed to drive class IV da neuron-specific expression (Zelle et al., 2013). The *ppk26* gene is predicted to encode a member of the DEG/ENaC family of ion channels, which are thought to be trimeric based on crystallographic evidence (Jasti et al., 2007). Each subunit contains two transmembrane helices flanking a large extracellular domain (Figure 1C). The closest relatives of Ppk26 in *Drosophila* are Ripped pocket (Rpk) and Pickpocket (Ppk1), as well as Ppk5, Ppk12, Ppk28, and Ppk29—all members of the DEG/ENaC family (Figure 1D).

A homology model of monomeric Ppk26, based on ASIC1 structure, demonstrates conservation of many structural features, including intracellular N and C termini, of 96 and 20 amino acids, respectively, two helical transmembrane domains, and a large extracellular loop structure comprising an arm and hand holding a ball (Jasti et al., 2007) (Figure 1C). Key conserved components of this extracellular loop are the wrist, palm, thumb,

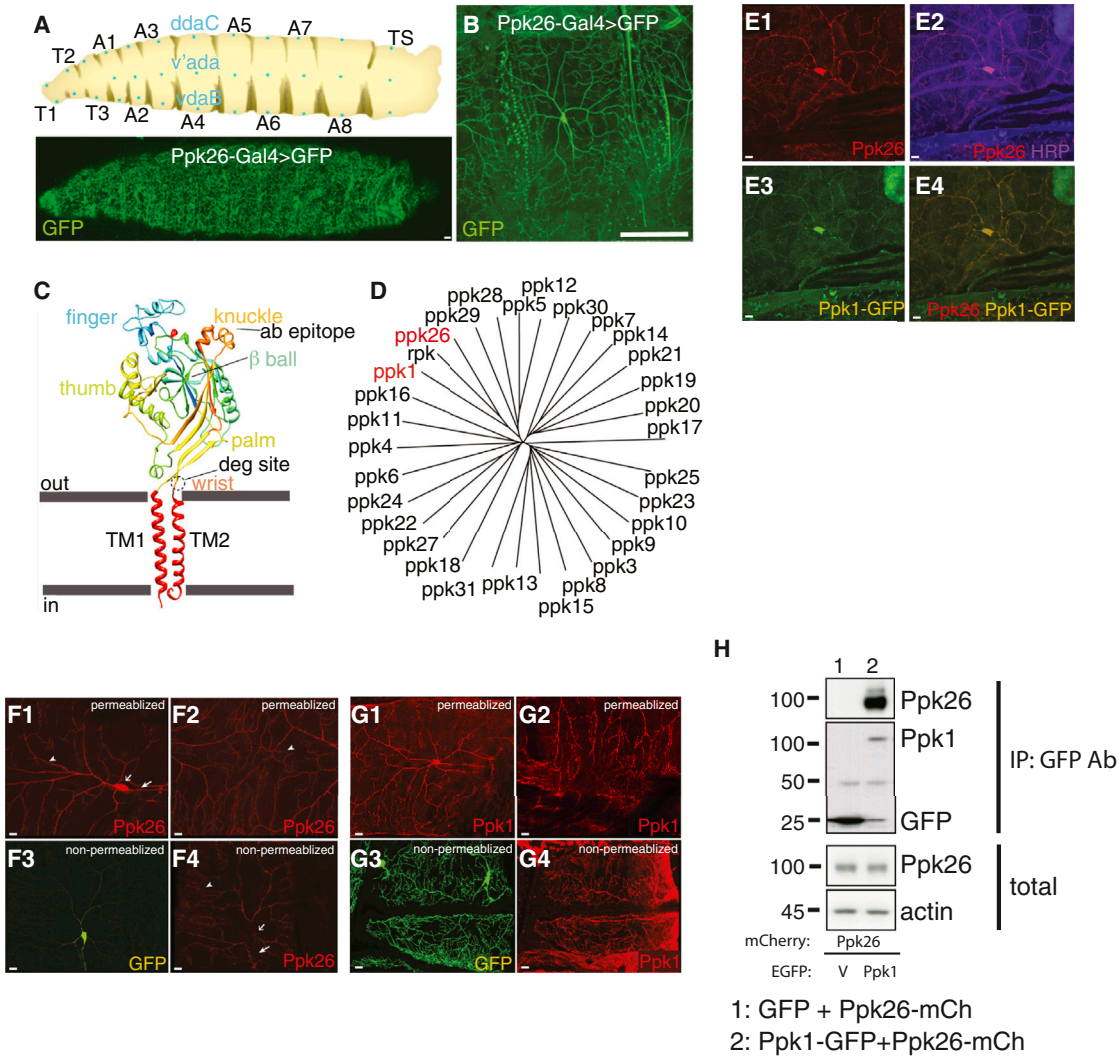

Figure 1. Ppk26 Is a DEG/ENaC Subunit Expressed in Class IV Neuron Dendrites and that Interacts with Ppk1

(A) Location of class IV neurons on the larval body wall.

(B) Expression pattern of *ppk26-Gal4* (Grueber et al., 2007). Scale bar represents 50 μm.

(C) Homology model of a single Ppk26 subunit based on the ASIC2A crystal structure. Peptide antibodies were raised against the knuckle region.

(D) Phylogenetic tree showing relationship between members of the Ppk family.

(E) Endogenous Ppk26 immunoreactivity was colocalized with Ppk1-EGFP immunoreactivity in class IV dendrites. Scale bar represents 10 μm.

(F) Ppk26 immunoreactivity was present in the cell body and dendrites of class IV neurons under permeablizing conditions (F1 and F2). Surface immunoreactivity was present throughout the dendritic compartment, including the proximal and distal dendrites (F3 and F4). Small arrowheads, terminal dendrites; open arrow, cell body; short arrow, axon.

(G) Ppk1 immunoreactivity was present in the cell body and terminal dendrites of class IV neurons (G1 and G2). Surface immunoreactivity was present throughout the dendritic compartment, including the proximal and distal dendrites. Scale bar represents 20 μm.

(H) Immunoprecipitation of EGFP from cells cotransfected with Ppk26-mCherry and Ppk1-EGFP or EGFP. Coprecipitation of Ppk26-mCherry was observed when cotransfecting with Ppk1-EGFP (lane 2), but not when cotransfecting with enhanced green fluorescent protein (EGFP) (lane 1; V, EGFP vector). Note that that immunoglobulin G heavy chain is observed at ~50 kDa in lanes 1 and 2 and that a EGFP degradation or cleavage product at ~27 kDa can be observed in lane 2. See also Figure S1.

finger, knuckle, and beta ball domains (Jasti et al., 2007) (Figure 1C). Conservation extends to the pore region of the predicted Ppk26 channel, where TM2 lines the pore and TM1 contacts the lipid bilayer (Gonzales et al., 2009; Baconguis and Gouaux, 2012) (Figure S1 available online). This conservation includes an alanine residue proximal to the pore that is thought to constitute the Degenerin site, namely, a residue that when mutated from alanine to valine leads to tissue degeneration, as well as increased channel open time and open probability in *C. elegans* (Brown et al., 2007). Thus, our screen identified Ppk26 as a DEG/ENaC ion channel expressed in class IV da neurons, which may form part of a functional channel with Ppk1.

Ppk26 Protein Is Specifically Expressed in Class IV da Neurons

To confirm that *ppk26-Gal4* reflects the endogenous expression pattern, we generated anti-peptide antibodies to a Ppk26 epitope (amino acids 532–539) located on the exposed and structurally rigid knuckle region (Figure 1C) (Jasti et al., 2007; Gonzales et al., 2009; Baconguis and Gouaux, 2012). In third-instar body walls, we found that anti-Ppk26 immunoreactivity is specifically localized to class IV da neurons in the peripheral nervous system (Figure 1E). Anti-Ppk26 signal was found in the cell body, as well as in the entire complement of dendritic processes, including higher and lower order branches, and in the axon initial segment (Figures 1F1 and 1F2). We also raised an antibody against a peptide (amino acids 506–523) inside the knuckle region of Ppk1. Similar to anti-Ppk26, an anti-Ppk1 signal was found exclusively in class IV da neurons—within the cell body, the entire complement of dendritic processes, and the axon initial segment (Figures 1G1 and 1G2). The specificity of both antibodies was validated by immunocytochemistry of Ppk1-EGFP and Ppk26-mCherry expressed in heterologous systems (Figure S1), as well as in null mutants (Figures 3A1–3A4).

ENaC channel surface trafficking is important for proper physiological function, and mutants affecting trafficking results in aberrant sodium transport, as is the case in the renal tubules of Liddle's syndrome patients (Snyder et al., 1995). However, very little is known about Ppk channel family localization in neurons, besides channel function in sensory bristles (Thistle et al., 2012) and motor neuron presynaptic terminals (Younger et al., 2013). To determine the likely site of Ppk26 function in class IV da neurons, we ascertained the cell-surface localization of the channel under permeabilizing versus nonpermeabilizing conditions (Figure 1F). Under permeabilizing conditions, Ppk26 was detected throughout the neuron, including the dendritic arbor (Figures 1F1 and 1F2). However, under nonpermeabilizing conditions, Ppk26 immunoreactivity was absent from the surface of the cell body and segments of primary dendrites proximal to the cell body (Figures 1F3 and 1F4). Instead, Ppk26 immunoreactivity was observed inside more distal regions of primary dendrites, as well as in lower order dendrites. Similarly, we found that under permeabilizing conditions, Ppk1 was detectable throughout the neuron, including the dendritic arbor (Figures 1G1 and 1G2). However, under nonpermeabilizing conditions, Ppk1 immunoreactivity was absent from the surface of the cell body and segments of primary dendrites proximal to the cell body. Instead, Ppk1 immunoreactivity was observed inside more distal regions of primary dendrites, as well as in lower-order dendrites (Figures 1G3 and 1G4). Thus, both Ppk26 and Ppk1 are specifically expressed in class IV da neurons and are inserted in the plasma membrane of dendrites at a distance from the cell body. However, the possibility that glial membrane wrapping of the soma (Han et al., 2011) may prevent antibody penetration to the soma cannot be ruled out.

Ppk26 and Ppk1 Likely Coexist in the Same Protein Complex

To test whether Ppk1 and Ppk26 could function together, we first examined if they had overlapping localization in class IV da neurons. Double labeling *ppk1* > Ppk1-EGFP-expressing animals with antibodies against GFP and Ppk26 revealed that both proteins are colocalized in these neurons (Figure 1E). We also performed immunoprecipitation experiments to determine if Ppk1 and Ppk26 are found in the same protein complex. We cotransfected HEK293 cells with either Ppk26-mCherry and Ppk1-EGFP or Ppk26-mCherry and EGFP as control and used anti-EGFP antibodies to immunoprecipitate proteins in complex with Ppk1-EGFP. We found that Ppk26 coimmunoprecipitated with Ppk1-EGFP when Ppk26-mCherry and Ppk1-EGFP were cotransfected (Figure 1H, lane 2), suggesting that these channel subunits can form a complex in heterologous cells. This coimmunoprecipitation was specific, because Ppk26 was not detectable in control immunoprecipitation from cells expressing Ppk26-mCherry and EGFP (Figure 1H, lane 1). The finding that Ppk26 can form a complex with Ppk1 and that both proteins colocalize in the same dendritic regions of class IV da neurons supports the notion that these channel subunits may assemble in vivo to form a functional heteromultimeric channel.

Interestingly, examination of RNA sequencing data through development (Daines et al., 2011) revealed that *ppk26* and *ppk1* transcripts have similar developmental expression dynamics (Figure S1C, bottom), with absolute *ppk26* RNA levels about twice that of *ppk1* RNA expression (Figure S1C, top).

Generation of *ppk26* and *ppk1* Mutants

Thus far, studies of Ppk1 in *Drosophila* have relied on analyses of a combination of RNAi and large deficiencies removing genes in addition to *ppk1* (Ainsley et al., 2003; Zhong et al., 2010; Boiko et al., 2012). To determine the function of Ppk26 and its relation to Ppk1, we generated specific *ppk26* (Figure 2B) and *ppk1* (Figure 2A) null mutants by "super-sized" Minos element excision, using the Minos-element strains MB01724 and MB10310, respectively, to generate specific deficiencies uncovering each gene (Witsell et al., 2009). We excised each of these Minos elements, which were located at the 3′ end of the *ppk26* or *ppk1* gene (Figure 2). Because imprecise excisions of Minos elements rarely generate large deletions, we combined respective Minos element strains for *ppk1* and *ppk26*, in a mutant background for *mus309*, the *Drosophila* homolog of Bloom syndrome helicase, to generate "super-sized" deletions. This *mus309* mutation interferes with double-stranded break repair, resulting in large and frequent deletions upon Minos element excision (Witsell et al., 2009). Using this strategy, we were able to generate large but specific excisions of each gene. Imprecise excision of MB01724 resulted in $Df(3R)ppk26^{rv11}$ and $Df(3R)ppk26^{rv29}$, referred to as *ppk26Δ11* and *ppk26Δ29.* The *ppk26Δ11* deletion removes the entire gene but retains a piece of the Minos element (Figure 2B), whereas *ppk26Δ29* removes all except for a piece of the first coding exon. To test for specificity, CG42458 and CG42660 located 1.4 kB upstream and 6.9 kB downstream, respectively, were assayed for genomic integrity (Figure S2A). Imprecise excision of MB10310 resulted in $Df(2L)ppk1^{rv5}$ and $Df(2L)ppk1^{rv16}$, referred to as *Ppk1Δ5* and *Ppk1Δ16*. The *Ppk1Δ5* deletion removes the gene through exon 7 UTR and retains a piece of the Minos element (Figure 2A), and *ppk1Δ16* removes the entire gene, also leaving behind a piece of the Minos element. To test for specificity, elbowB and spel1 genes, located 9.4 kB upstream and 11.3 kB downstream of *ppk1*, respectively,

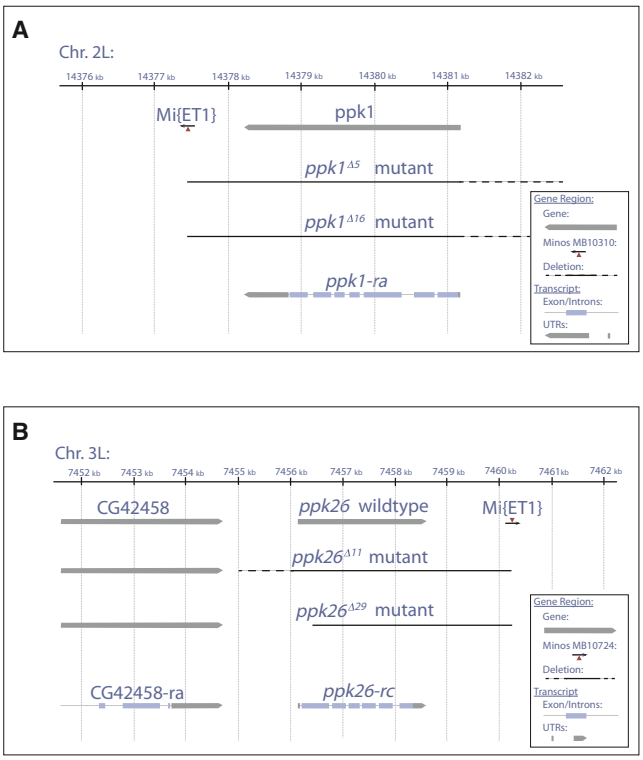

Figure 2. Generation of Specific ppk1 and ppk26 Null Mutants

(A) Schematic showing excision of the Mi{ET1}MB10310 element at the 3′ of ppk1/CG3478 produced *ppk1Δ5* and *ppk1Δ16* mutants, which delete the entire open reading frame of Ppk1, leaving behind a piece of the Mi{ET1} element.

(B) Schematic showing excision of the Mi{ET1}MB10724 element at the 3′ of *ppk26*/CG8546 gene produced *ppk26Δ11* and *ppk26Δ29* alleles. *ppk26Δ11* deletes the entire open reading frame of *ppk26*, leaving behind a piece of the Mi{ET1} element. *ppk26Δ29* deletes exons 3 through 6, as well as the 3′ UTR and polyadenylation sequence, keeping intact the noncoding exon 1 and part of the coding exon 2. Dashed lines indicate the region containing the break point, which does not affect upstream or downstream genes. A solid line indicates deletions confirmed by PCR.

See also Figure S2.

were assayed for integrity in each case. Both genes were found to be intact (Figure S2B). As genetic background controls, we also isolated precise excisions of MB01724 and MB10310, resulting in the revertants Mi{ET1}MB10724^{rv214} for Ppk26 and Mi{ET1}MB10310^{rv1} for Ppk1. These specific null alleles of *ppk26* and *ppk1* will be useful for future functional studies of these two coexpressed DEG/ENaC channel subunits, as well as analysis of genetic interactions.

Ppk1 and Ppk26 Exhibit Mutual Dependence for Dendritic Plasma Membrane Localization

Ion channels and receptors composed of multiple subunits are often assembled in the endoplasmic reticulum (ER), and traffic as multimers to their site of function (Heusser and Schwappach, 2005; Muth and Caplan, 2003). Thus, lack of one of the subunits may prevent the others from reaching their destination. For example, the vanilloid receptor (TRPV) channel subunits, Nanchung and Inactive, which are critical for hearing in the adult fly (Gong et al., 2004) and larval sound response (Wu et al., 2011; Zhang et al., 2013) have interdependent trafficking in chordotonal organs. Mutants in either *nanchung* or *inactive* result in the apparent absence of the other subunit in cilia, possibly accounting for the hearing defect in either mutant (Gong et al., 2004), and indicating that expression is either coregulated or that the proteins become degraded in single mutants. In *C. elegans*, OSM-9 and OCR-2 channels also have mutually dependent transport and function in the ciliated ASH neuron (Tobin et al., 2002), although surface expression has not been assayed.

If Ppk1 and Ppk26 form part of the same ion channel, then their trafficking to the cell surface might be linked. To test this hypothesis, we examined the surface expression of Ppk26 on the dendritic plasma membrane under nonpermeabilizing conditions, in both wild-type larvae and *ppk1Δ16* mutants. Strikingly, in the absence of Ppk1, Ppk26 failed to be inserted at the plasma membrane of dendrites (Figures 3B1 and 3B2). Similarly, we found that the removal of Ppk26 prevented Ppk1 from localizing to the plasma membrane of dendrites (Figures 3B3 and 3B4). Thus, Ppk1 and Ppk26 are mutually required for localization to the surface of dendrites, presumably their site of function. Interestingly, although surface localization was mutually dependent, under permeablizing conditions both Ppk1 and Ppk26 immunoreactivities were still found in the dendrites of Ppk26 and Ppk1 mutants (Figures 3C and 3D), suggesting that it is plasma membrane integration that is affected. Although Ppk1 and Ppk26 are exclusively expressed in class IV da neurons, we also confirmed that other da neuron classes were present in *ppk26* and *ppk1* mutants by labeling preparations with Futsch/22C10 antibody, which marks a neuronal epitope (Figures 3C and 3D).

Ppk26 Degenerin Mutation Leads to Loss of Class IV da Neuron Dendrites

Mutations leading to increased activity of the human ENaC channel result in rare dominant hereditary diseases that impact kidney, colon, and lung tissues (Kellenberger and Schild, 2002). In MEC channels, it has been suggested that Degenerin mutations (substitution at the Degenerin residue; alanine for threonine, valine, or aspartate) increases calcium permeability (Bianchi et al., 2004), as well as channel open time and open probability (Brown et al., 2007), thereby increasing cytoplasmic calcium levels and leading to excitotoxicity. However, a degenerin phenotype has not been reported for Ppk channels, including animals overexpressing Ppk1-Deg (Wegman et al., 2010). Because Ppk26 contains a conserved Degenerin residue (Figure 1C), we sought to ascertain the effects of this mutation in class IV da neurons. In particular, we tested a Ppk26-Deg-mCherry transgene containing the Degenerin mutation (A547V) in the peri-TM2 region of the protein for expression in class IV neurons. Overexpressing a wild-type Ppk26-mCherry transgene alone in class IV da neurons did not result in any obvious abnormal phenotypes in these neurons (Figure 4A). In contrast, expressing a Ppk26-Deg-mCherry mutant transgene resulted in loss of dendritic coverage by many of the class IV da neurons along the body wall (Figure 4B), due to a drastic reduction in both primary and secondary dendrites (Figure 4C). This diverged from the observation that, as previously reported (Wegman et al., 2010),

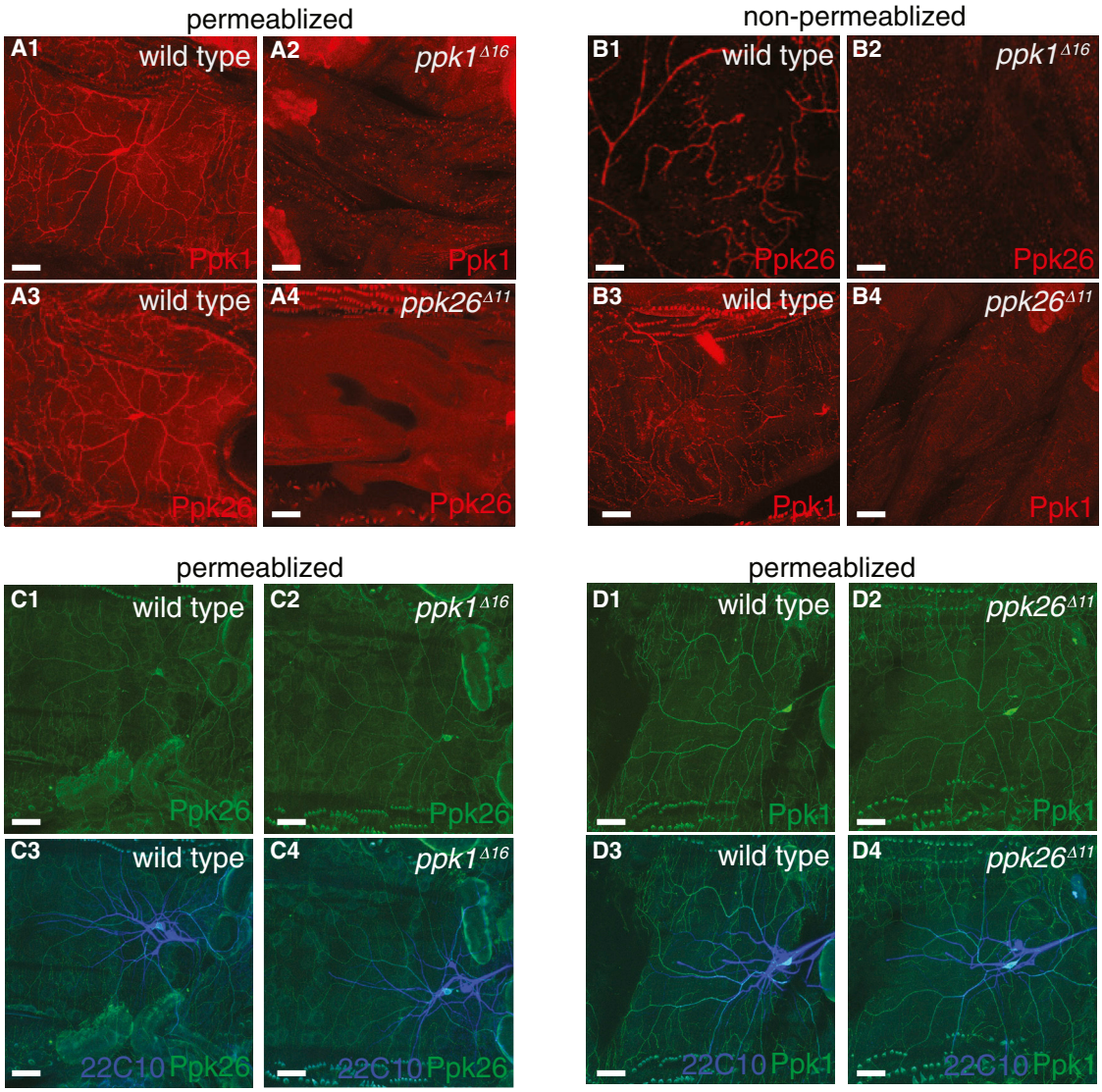

Figure 3. Ppk1 and Ppk26 Show Mutually Dependent Surface Expression on Class IV Neuron Dendrites

(A) ppk1 and ppk26 null mutants lack immunoreactivity under permeablizing conditions.

(B) Ppk26 was present on the surface of class IV neuron dendrites in wild-type and absent or severely reduced in mutants lacking Ppk1 (B1 and B2). Ppk1 was present on the surface of class IV neuron dendrites in wild-type and absent or severely reduced in mutants lacking Ppk26 (B3 and B4).

(C) Immunostaining under permeablizing conditions showed that mutants lacking Ppk1 still contained Ppk26 in class IV neuron dendrites.

(D) Immunostaining under permeablizing conditions showed that mutants lacking Ppk26 still contained Ppk1 protein at class IV dendrites. In (C) and (D), Futsch/22C10 immunostaining confirmed the presence of the remaining neurons in the da neuron cluster in both wild-type and mutants. Scale bar represents 30 μm.

expressing the Ppk1-Deg mutant did not elicit a strong dendritic arbor phenotype (Figure S4).

Ppk26 Regulates Locomotor Behavior

Class IV da neurons are implicated in locomotor behavior, presumably by acting as proprioceptors that modulate motor output in a Ppk1-dependent manner (Ainsley et al., 2003). To test whether Ppk1 and Ppk26 regulate locomotor behavior via their activity in class IV da neurons in a cell-autonomous manner, we examined locomotor behavior in larvae expressing either Ppk26-RNAi or Ppk1-RNAi in class IV da neurons using the *ppk1-Gal4* driver (Grueber et al., 2007). Crawling behavior in *Drosophila* larvae consists of sequential contractions of circumferential and longitudinal muscles in each segment, which propagate from posterior to anterior segments (Fox et al., 2006; Hughes and Thomas, 2007; Song et al., 2007; Ainsley et al., 2003; Vogelstein et al., 2014). Knockdown of either Ppk1 or Ppk26 resulted in a decrease in turning frequency (Figure 5A), such that locomotion was predominately composed of directional crawling. Similar abnormal behavior was observed in *ppk1* and *ppk26* null mutants (Figures 5B and 5E), but not in their respective revertants. This defect was rescued by expressing a

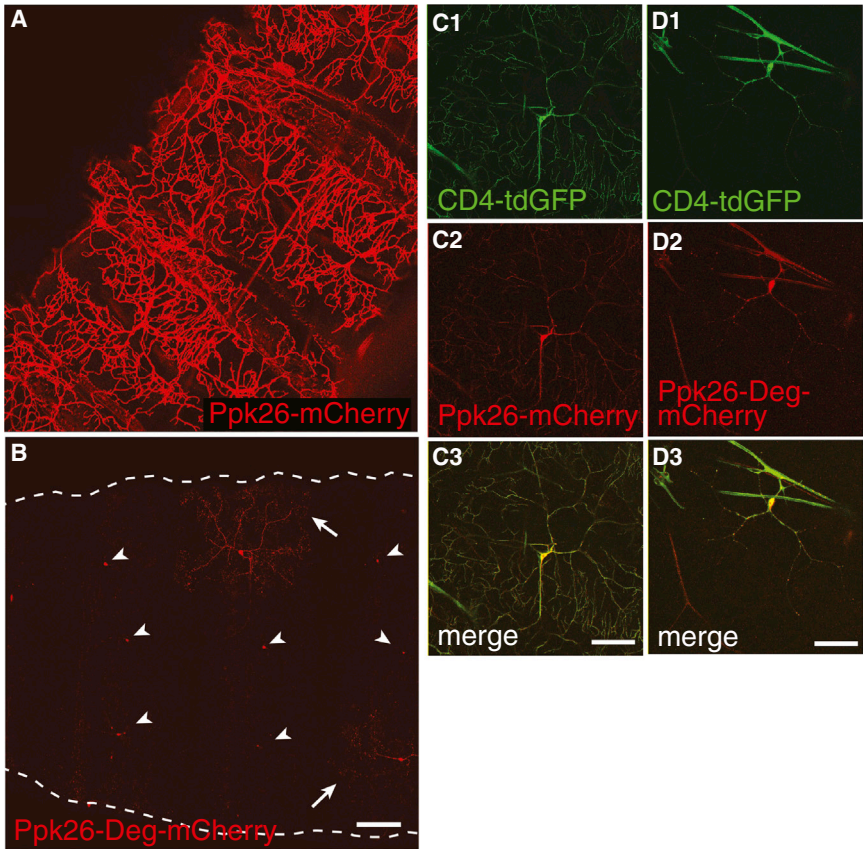

Figure 4. Overexpression of Ppk26-Degenerin Results in a Reduction in Class IV Dendritic Arbors

(A) Overexpression of Ppk26-mCherry in class IV neurons using the Ppk1 promoter resulted in strong localization to dendrites with elaborate primary and higher-order arbors.

(B) Overexpression of Ppk26-degenerin-mCherry resulted in a dramatic reduction in dendritic arbors of midsecond instar larvae. Arrowheads, cell bodies; arrows, fragmented and reduced dendritic arbors. Scale bar represents 300 μm.

(C and D) Overexpression of Ppk26-degenerin-mCherry in a wild-type background resulted in reduced arbor complexity of third-instar class IV neurons, as evidenced by the membrane marker CD4-tdGFP. Scale bar represents 60 μm.

wild-type Ppk1-EGFP or Ppk26-mCherry transgene in class IV neurons of the mutants (Figures 5C and 5E), thus confirming the critical role of these channel subunits in controlling locomotion. To look for any additive effect, we examined the phenotype of *ppk1; ppk26* double mutants and found that turning was reduced to a similar extent as the single null mutant of either *ppk1* or *ppk26* (Figure 5D), supporting the notion that the two genes function together in the same pathway. Silencing the class IV neurons by overexpressing Kir2.1 showed a reduction in turning, consistent with class IV sensory neurons themselves contributing to this locomotion behavior (Figure 5G). Interestingly, *ppk1* and *ppk26* transheterozygotes did not differ from wild-type controls (Figure 5H).

Because Piezo is a mechanosensitive channel required in class IV da neurons for harsh mechanical touch sensation (Kim et al., 2012), we also examined turning behavior in these mutants. In contrast to *ppk1* and *ppk26* mutants, there was no significant difference in *piezo* mutant-turning behavior (Figure 5F). Because *painless* and *dTrpA1* have also been implicated in class IV da neuron function, we also tested mutations in these genes for turning behavior. Because a bona fide null allele for Painless was not available, we generated $pain^{pc}$ and $pain^{pf}$ alleles missing the coding region and the entire gene region, respectively (Figure S3), to be used in conjunction with existing alleles. Whereas *dTrpA1* mutants showed no significant defect in locomotion, $pain^{1}$, $pain^{pc}$, and $pain^{pf}$ alleles showed significant defects in turning behavior (Figure 5F). While $pain^{1}$ showed defects in both thermal and mechanical nociception, $pain^{pc}$ and $pain^{pf}$ mutants showed less severe phenotypes (Figure S6).

Ppk26 Serves Mechanical, but Not Thermal, Nociception Behavior

Class IV neurons have been implicated in both proprioceptive and multimodal nociceptive behaviors. Because class IV neurons are important for thermal nociception behavior (Chattopadhyay et al., 2012; Hwang et al., 2012), we examined thermal response in mutant animals. While animals expressing Kir2.1 in class IV neurons had a significant rightward shift in temperature response to a 46°C heated probe as compared to wild-type (Figures 6D–6G), *ppk1* and *ppk26* mutants showed no significant difference from wild-type (Figures 6A–6C). Because Ppk1, as well as Piezo, have been implicated in class IV da neurons for harsh touch sensation (Kim et al., 2012), we also tested harsh touch behavior in *ppk1*, *ppk26*, and *piezo* mutants. We found significant defects in harsh touch behavior in all of the above mutants, consistent with previous work, as well as our finding that Ppk26 is the likely partner of Ppk1.

DISCUSSION

We have identified Ppk26, an DEG/ENaC channel subunit that is coexpressed with Ppk1in class IV da neurons. Consistent with the model that Ppk26 and Ppk1 may be subunits of the same channel, we found that Ppk1 and Ppk26 colocalize in class IV da neurons, they form a complex in heterologous expression systems, and they show nonadditive and nonredundant mutant phenotypes in vivo. Ppk26 protein was found in somatic, dendritic, and axonal compartments, and plasma membrane insertion was observed in terminal dendrites. Ppk1 and Ppk26 were reciprocally required for normal trafficking and/or insertion to the plasma membrane, further supporting the notion that these two channel subunits interact in vivo. We show that, as is the case for MEC Degenerin channels, mutations at the Degenerin position of Ppk26 lead to loss of class IV da neuron integrity.

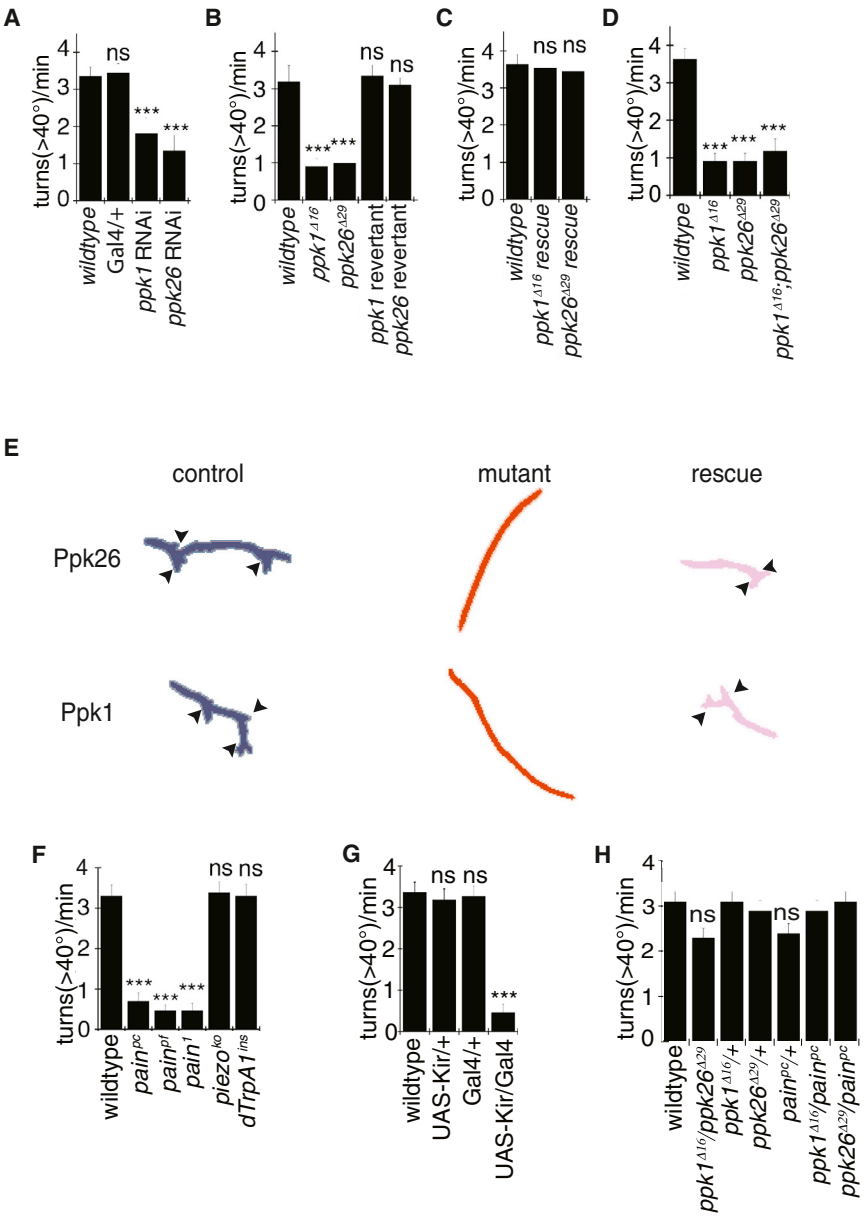

Figure 5. Ppk26 and Ppk1 Are Required for Locomotion Behavior

(A) RNAi-mediated knockdown of Ppk26 or Ppk1in class IV neurons with *ppk1-Gal4* driver (Grueber et al., 2007) results in reduced turn frequency.

(B) *ppk1* and *ppk26* null mutant, but not revertant strains, have a reduced frequency of turns.

(C) Expression of Ppk1-EGFP or Ppk26-mCherry in class IV neurons, using one copy of a direct *ppk1* promoter fusion, rescued the turning defects in *ppk1* and *ppk26* mutants.

(D) *ppk26* and *ppk1* mutants had a reduced turning frequency.

(E) Representative traces of wild-type, mutant, and rescue animals for Ppk26 and Ppk1. Arrowheads, turning.

(F) Locomotor turning behavior of *painless*, *dTrpA1*, and *piezo* mutants.

(G) Effect of silencing class IV neurons.

(H) Turning behavior of heterozygotes and trans-heterozygotes. Error bars denote ± SEM; n ≥ 10; *** $p < 0.001$; Student's t test.

See also Figure S5.

We also found that Ppk26 function plays essential roles in normal larval locomotion, particularly in turning behavior.

Proposed Role of Ppk1 and Ppk26 in Mechanosensation by Class IV da Neuron Dendrites

Overexpression of EGFP-tagged MEC channels in *C. elegans* has been reported to result in a punctate localization, leading to suggestions that each of these puncta represents a mechanosensory apparatus (O'Hagan et al., 2004). In light of the speculation that contact of dendrites with subcuticular epidermis is part of the apparatus that senses mechanical stimuli (Chalfie 2009; Chen and Chalfie, 2014; Han et al., 2012), it is intriguing that we find both Ppk1 and Ppk26 on the surface of distal and higher-order dendrites (Figure 1), consistent with channel function in this compartment. Mutants in Ppk1 and Ppk26 showed defects in the frequency of turning of freely crawling larvae (Figure 5). Moreover, loss of function of either or both Ppk26 and Ppk1 had the same effect on larval locomotion. These findings support the notion that Ppk26 and Ppk1 may act in the same pathway—perhaps in the same channel complex—in mechanosensation that is important for proper locomotion.

Is Ppk26 Associated with Ppk1 in a Multimeric Channel?

Class IV da neurons are known to express the DEG/ENaC channel subunit Ppk1 (Adams et al., 1998), an observation that was confirmed in our study. Given that DEG/ENaC channels are trimeric ion channels that typically require the assembly of different subunits for proper function, the specific coexpression of Ppk26 in class IV da neurons raises the possibility that Ppk1 and Ppk26 may correspond to different subunits of the same mechanosensory channel in vivo. This is supported by our findings of biochemical interactions between Ppk1 and Ppk26 (Figure 1), their null mutant phenotypes that indicate nonredundancy (Figure 5), and by the reciprocal requirements of Ppk1 and Ppk26 for normal trafficking or insertion to the plasma membrane (Figure 3). The mechanism for this mutual dependence is currently unknown.

Notably, inspection of the developmental expression profile of *ppk1* RNA and *ppk26* RNA revealed a similar time course, with the absolute levels of RNA for *ppk26* twice as high as those of RNA for *ppk1* throughout development (Figure S1). Although we cannot be certain that this quantitative difference in RNA levels reflects a similar quantitative difference in the levels of

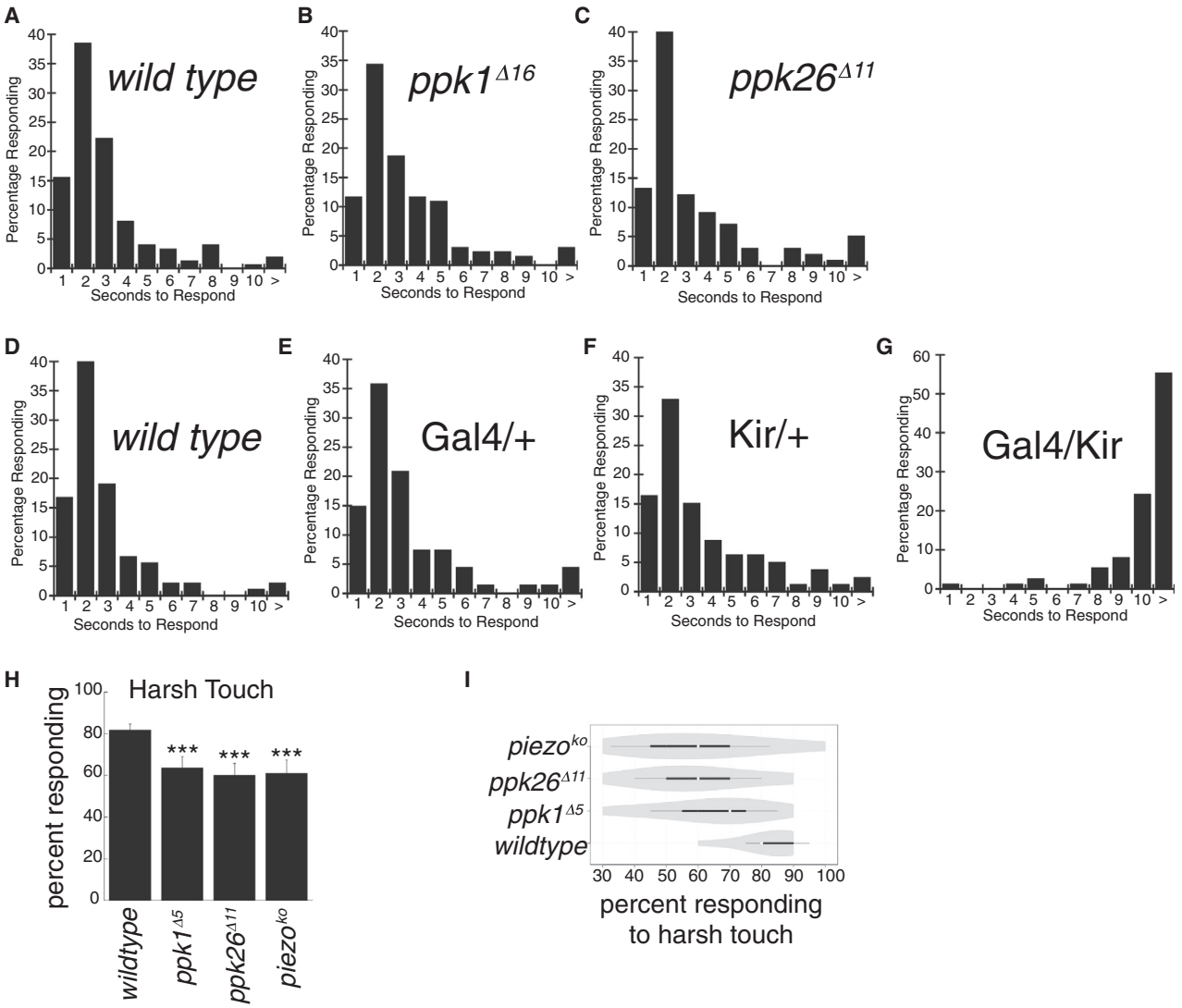

Figure 6. Ppk26 and Ppk1 Are Required for Mechanical, but Not Thermal, Nociception

(A–C) Thermal nociception of *ppk1* and *ppk26* mutants were assessed by applying a thermal probe and monitoring the time to nocifestive response ($n \geq 100$). (D–G) Silencing of class IV neurons using ppk-Gal4 (Grueber et al., 2007) resulted in a strong defect in thermal nociception response time ($n \geq 100$).

(H and I) *ppk1*, *ppk26*, and *piezo* mutant animals showed significant defects in harsh mechanical nociception response. Error bars ± SEM; $n \geq 10$; *** $p < 0.001$; Student's t test and Fisher's exact test. In violin plots, white circles show the medians, box limits indicate the 25th and 75th percentiles as determined by R software, whiskers extend 1.5× the interquartile range from the 25th and 75th percentiles, and polygons represent density estimates of data and extend to extreme values. See also Figure S6.

Ppk1 and Ppk26 subunits, it is tempting to speculate that two Ppk26 subunits and one Ppk1 subunit may be assembled to form a surface-expressed trimeric channel.

Tendency of the Degenerin Mutation to Be Involved in Neuronal Degeneration

Studies in many systems have suggested that mutations in the Degenerin domain of DEG/ENaC lead to loss of cell integrity and perhaps degeneration; however, this behavior has not yet been observed in *Drosophila* Pickpocket family members. The Degenerin position is localized in the wrist region, close to the mouth of the pore. Thus, it has been suggested that Degenerin mutations change the properties of the channel, increasing open time probability and perhaps shifting its ion selectivity from Na^+ to Ca^{2+} (Bianchi et al., 2004; Brown et al., 2007). Given the tight regulation of Ca^{2+} within cells and its involvement in critical cellular processes, this increase in Ca^{2+} permeability may lead to loss of cellular homeostasis, in a process that has been dubbed excitotoxicity. Consistent with these findings, we also observed that when a Degenerin mutation was introduced into Ppk26 that was overexpressed in class IV da neurons, it resulted in a marked reduction in dendritic arbor size (Figure 5). This suggests that the Ppk26-Deg mutation leads to toxicity in the sensory neurons, as has been observed in DEG/ENaC channels in

C. elegans, but not in Ppk1 (Wegman et al., 2010). Whereas it is likely that the function of the pore structure of these channels is evolutionarily conserved, it is unclear why Ppk26-Deg has a more potent effect than Ppk1-Deg. One possibility is that the Ppk26-Deg residue makes a larger contribution to pore structure than Ppk1-Deg, because of its intrinsic structure or because of a potential 2:1 stoichiometry in vivo.

Role of Ppk26 and Ppk1 in Larval Locomotion

Larval locomotion is likely regulated by sensory input provided by sensory neurons in the body wall, which may in turn modulate the motoneurons innervating the body wall. The *C. elegans* mechanosensitive TRPN channel TRP-4 acts in the DVA neuron to coordinate bending behavior and body posture through positive and negative modulation (Li et al., 2006). It seems likely that class IV da neurons likewise provide some information for sensory modulation of locomotion through a mechanosensory mechanism, but future work will need to determine if this is indeed the case.

Role of Class IV da Neurons in Proprioception

Proprioception is a mechanosensory process involving sensory neurons that transduce the mechanical information related to body position or characteristics of the environment for the generation of appropriate behavioral output, such as the turning locomotor behavior that is essential for foraging larvae (Chalfie, 2009; Proske and Gandevia, 2012). Here, we have identified a member of the DEG/ENaC family of proteins, Ppk26, which acts together with Ppk1 likely as subunits of a channel important for mechanosensation. Our results suggest that perhaps a major site of mechanosensory transduction is located in class IV da neuron dendritic processes. The behavioral phenotypes of larvae with Ppk1 and Ppk26 knockdown in class IV da neurons, as well as the respective null mutants or double mutants, suggest that a deficit in these channels interferes with the ability of the animal to execute proper turning behavior, raising the possibility that the two subunits could be involved in proprioceptively sensing the deformation of the cuticle. Whether class IV neurons function as proprioceptors still needs to be directly demonstrated, and future experiments will be needed to address the relationship between class IV neural activity and body position.

Role of Class IV da Neurons in Nociception

Numerous studies have implicated the class IV da neurons in both thermal and mechanical nociception behavior (Zhong et al., 2010; Zhong et al., 2012; Kim et al., 2012). We found that whereas Ppk1 and Ppk26 are important for mechanical nociception behavior, they are dispensable for thermal nociception behavior. While Ppk1 and Ppk26 channels indeed moonlight during two processes in the same neuron, namely, mechanical nociception and proprioception, these channels must be playing a specific role as they are not involved in thermal response by the same neuron (Figures 4 and 6).

Different Ion Channels May Serve Different Mechanosensory Modalities in the Same Neuron

Whereas the class I da neurons and bd neurons are implicated in proprioception for the regulation of sequential contractions use the TRPN channel NompC as the sensor (Cheng et al., 2010), class IV da neurons rely on the DEG/ENaC channel likely composed of Ppk26 and Ppk1 for the regulation of turning behavior as well as mechanical nociception, perhaps through sensing a mechanical signal at the cuticle. Interestingly, we found that Piezo, a bona fide mechanotransducing ion channel involved in class IV da neuron mechanotransduction and required for mechanical nociception, does not appear to be involved in turning behavior, suggesting that different combinations of ion channels may serve different mechanosensory functions in the same neuron.

EXPERIMENTAL PROCEDURES

For more details regarding the procedures, see the Supplemental Experimental Procedures.

Molecular Biology and Cloning

Standard cloning techniques were used to generate the C-terminal tagged Ppk26-mCherry and Ppk1-EGFP. Ppk1 > Ppk26-mCherry and Ppk1 > Ppk1-EGFP lines used for rescue experiments were cloned into an APPHIH vector containing the Ppk enhancer. UAS expression constructs were generated by cloning the tagged genes into pTW and pUAST. UAS-Ppk26 Degenerin-mCherry was generated by site-directed mutagenesis (A547V) of Ppk26-mCherry/pmCherry-N1.

Locomotion Behavior Assay

Wandering third-instar larvae were placed on a fresh agarose plate, equilibrated for 2–3 min, and then transferred to an additional agarose plate for imaging. The plate was illuminated tangentially using 850 nm infrared LED strips 120° beam angle, #3258 (http://www.environmentallights.com). The LED light was passed through a diffuser to form an arena with high contrast. The larvae were tracked using OpenCV software.

Mechanical and Thermal Nociception Assays

A mechanical nociception assay was performed as in Zhong et al. (2010). Each trial corresponds to the percentage of responding larvae. A thermal nociception assay was performed as in Hwang et al. (2012).

Immunocytochemistry and Immunofluorescence Imaging

Larvae were dissected in PBS and fixed in 4% paraformaldehyde. For permeablizing conditions, larvae were washed with 0.1% Triton X-100 in PBS, and for nonpermeablized conditions, Triton X-100 was omitted. Rabbit anti-Ppk26 was used at 1:10,000, and rabbit anti-Ppk1 was used at 1:3,000.

Antibody Generation

Rabbit anti-peptide antibodies were generated against synthetic peptides representing the knuckle region of PPK1 (AA 506-523) and PPK26 (AA 532-539) (YenZym).

Fly Genetics and Mutant Generation

The following fly stocks were used: Mi{ET1}MB10724, Mi{ET1}MB10310, P{hslLMiT}2.4, mus309[N1], and mus309[D2] (Bloomington Stock Center). UAS-Ppk26-mCherry, Ppk1>Ppk26-mCherry, UAS-Ppk1-EGFP, and Ppk1>Ppk1-EGFP were generated in the lab via germline transformation. *ppk1-RNAi* was obtained from Dr. Fen-Biao Gao (Xu et al., 2004). *ppk26*-RNAi (GD5110) was obtained from VDRC. $pain^{pc}$ and $pain^{pf}$ were generated as in Huang et al., 2009. $dTrpA1^{ins}$ was obtained from Dr. Paul Garrity, and $piezo^{ko}$ was obtained from Dr. Ardem Patapoutian. A 1 kB *ppk1*-Gal4 was used to label class IV neurons (Grueber et al., 2007). Mi{ET1} excisions were performed in a *mus309* mutant background as in Witsell et al. (2009).

Immunoprecipitation and Westerns

Immunoprecipitations were performed from transfected HEK293 cell homogenates using protein A-conjugated Sepharose beads (Invitrogen). Proteins

separated by SDS-PAGE were transferred to nitrocellulose membranes and after immnolabeling examined by chemiluminescence.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.10.034.

AUTHOR CONTRIBUTIONS

D.A.G., Y.N.J., and L.Y.J. designed the experiments. D.A.G., S.Y., S.E.K., L.C., H.Y.L., and W.S. all contributed to the experiments. S.Y. and D.A.G. made the ppk1 and ppk26 mutants. S.Y., S.M., and L.C. made the painless mutants. D.A.G. and S.M. performed the behavioral experiments and analyzed the data. W.S. made ppk1 transgenic lines and ppk26-Gal4. D.A.G. made ppk26 transgenic lines. D.A.G. and Y.N.J. wrote the paper.

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