

Balboa Binds to Pickpocket In Vivo and Is Required for Mechanical Nociception in *Drosophila* Larvae

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Summary

The *Drosophila* gene *pickpocket* (*ppk*) encodes an ion channel subunit of the degenerin/epithelial sodium channel (DEG/ENaC) family [1]. PPK is specifically expressed in nociceptive, class IV multidendritic (md) neurons and is functionally required for mechanical nociception responses [2, 3]. In this study, in a genome-wide genetic screen for other ion channel subunits required for mechanical nociception, we identify a gene that we name *balboa* (also known as *CG8546*, *ppk26*) [4]. Interestingly, the *balboa* locus encodes a DEG/ENaC ion channel subunit highly similar in amino acid sequence to PPK [5]. Moreover, laser-capture isolation of RNA from larval neurons and microarray analyses reveal that *balboa* is also highly enriched in nociceptive neurons. The requirement for Balboa and PPK in mechanical nociception behaviors and their specific expression in larval nociceptors led us to hypothesize that these DEG/ENaC subunits form an ion channel complex in vivo. In nociceptive neurons, Balboa::GFP proteins distribute uniformly throughout dendrites but remarkably localize to discrete foci when ectopically expressed in other neuron subtypes (where PPK is not expressed). Indeed, ectopically coexpressing *ppk* transforms this punctate Balboa::GFP expression pattern to the uniform distribution observed in its native cell type. Furthermore, *ppk-RNAi* in class IV neurons alters the broad Balboa::GFP pattern to a punctate distribution. Interestingly, this interaction is mutually codependent as *balboa-RNAi* eliminates Venus::PPK from the sensory dendrites of nociceptors. Finally, using a GFP-reconstitution approach in transgenic larvae, we directly detect in vivo physical interactions among PPK and Balboa subunits. Combined, our results indicate a critical mechanical nociception function for heteromeric PPK and Balboa channels in vivo.

Results and Discussion

Using a set of UAS-mediated RNAi lines that covers the majority of genes encoding ion channels in the genome [6], we

carried out a tissue-specific RNAi screen to identify channels required for behavioral responses to noxious mechanical force [4]. Noxious force is detected by *Drosophila* larvae primarily through activation of the nociceptive class IV multidendritic (md) neurons, but other classes of md neurons also contribute to noxious force detection, albeit to a lesser degree [7]. Thus, to identify ion channels required for noxious force detection, we first crossed our UAS-RNAi collection [4, 6] to the *md-GAL4* driver [4, 8], which drives expression in all classes of md neurons, and then tested the larval progeny for normal or abnormal nocifensive rolling responses to stimulation with a 50 mN Von Frey fiber [4, 8]. This primary screen yielded defective responses in UAS-RNAi lines targeting nine channel subunits [4]. In parallel studies, we investigated the genome-wide expression levels of mRNAs isolated from class IV and class I md neurons by performing laser-capture microdissection of the target fluorescently labeled neurons from cryosectioned larvae. The class IV samples showed high enrichment of *pickpocket* (*ppk*), *Gr28b*, and *knot* transcripts that are known to have enriched expression in these cells [1, 9, 10] (Figure 1A). As expected, the transcript for the panmultidendritic neuron marker *multiple* [11] was seen in both class IV and class I samples. Remarkably, we found highly enriched expression in the class IV neurons (relative to the class I neurons) for an mRNA that encoded one of the ion channel subunits that was identified in our forward genetic screen (*CG8546/ppk26*) (Figure 1A). In addition to the microarray evidence, a *ppk26-GAL4* reporter gene [5] drove expression of *UAS-mCD8::GFP* exclusively in the class IV md neurons (Figures 1B and 1C). Interestingly, *CG8546* is highly similar in amino acid sequence to PPK [5], a degenerin/epithelial sodium channel (DEG/ENaC) previously shown to be expressed in class IV neurons that is required for mechanical nociception [1, 2].

Given the highly enriched expression in the nociceptive class IV neurons, we tested the effects of knocking down *CG8546* specifically in these cells. To do so, we crossed the UAS-RNAi lines targeting *CG8546* to the class IV-specific *ppk-GAL4* driver. Consistent with the highly enriched class IV expression pattern that was detected in our microarray, RNAi targeting of *CG8546* with the class IV driver nearly eliminated the transcript from whole-animal RNA isolates (Figures S1A and S1B available online). In addition, we found that *CG8546* knockdown in the class IV neurons resulted in profound mechanical nociception behavioral defects (Figure 1D). Thus, to reflect this defective mechanical nociception phenotype, we named the gene *balboa* (*bba*), in honor of the fictional pain-resistant prizefighter hero, Rocky Balboa [4]. As previously described for *ppk* [2], expressing *balboa-RNAi* in the class IV neurons did not cause a defect in optogenetically triggered nociception behaviors (Figure 1E) [4]. Therefore, the effects of *balboa-RNAi* are unlikely to be explained by a nonspecific effect on the intrinsic excitability or general health of the class IV neurons. Indeed, *balboa-RNAi* did not cause a noticeable change in class IV neuron morphology (Figures S1C and S1D). Given the well-established role for ion channels of this gene family in *C. elegans* mechanotransduction [12], an interesting possibility is that Balboa is also involved in force sensing by the class IV cells.

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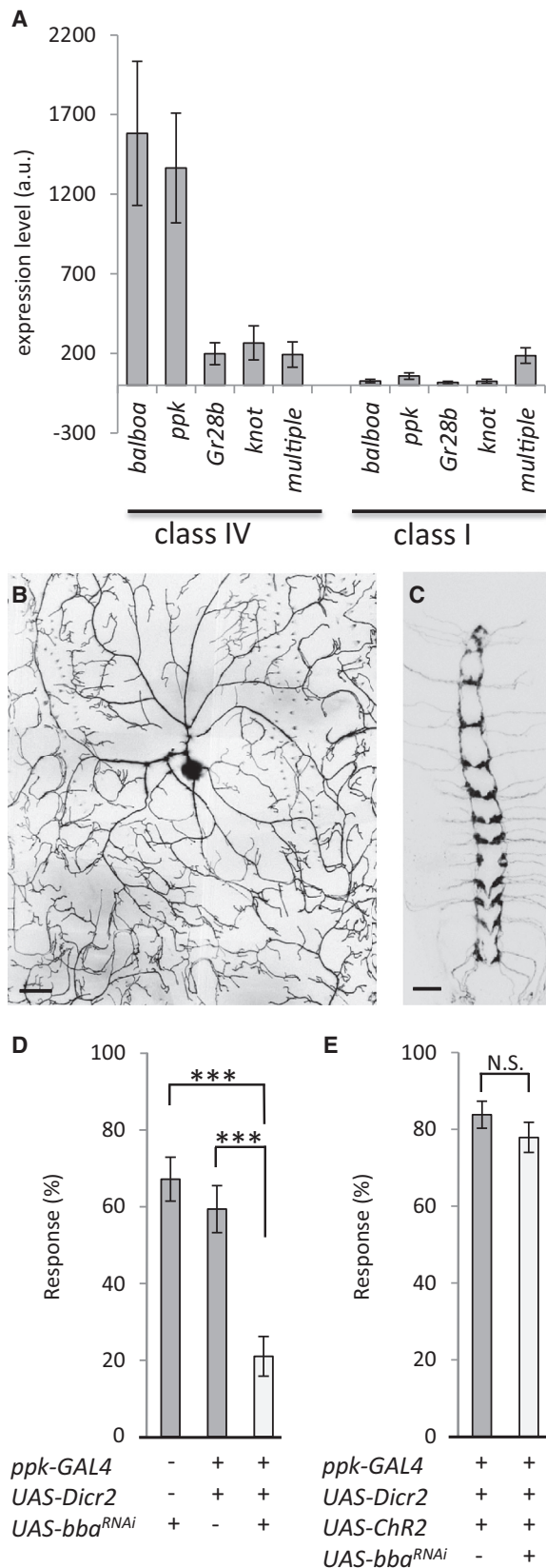


Figure 1. Balboa Is Highly Expressed in Nociceptive Neurons and Is Required for Mechanical Nociception

(A) Affymetrix microarrays performed on mRNA isolated from class IV and class I neuron cell bodies show high enrichment of *balboa*, *ppk*, *Gr28b*,

We next investigated the subcellular localization of the Balboa protein by creating transgenic flies expressing a GFP-tagged version of Balboa (*UAS-balboa::GFP*). When crossed to the *md-GAL4* driver, the subcellular localization of Balboa::GFP was observed to differ between the various classes of md neurons (Figure 2A). Within the class IV neurons, we observed uniform Balboa::GFP fluorescence throughout the dendritic arbor, but in the class I, class II, and class III neurons (where *balboa* may not normally be expressed), Balboa::GFP localized to bright punctae within the dendritic arbors (Figure 2B). Although these punctae appeared similar to those previously described for expression of MEC-4 in *C.elegans* mechanosensory neurons [13], it is likely that these ectopic punctae are distinct and represent an intermediate stage of Balboa::GFP protein trafficking that is not surface localized or is aggregated. Using drivers specific to different classes of neurons, we confirmed that the uniform distribution of Balboa was due to its presence in the class IV neurons where it is endogenously expressed (Figures 2C and 2D) and that the highly punctate distribution was due to its ectopic expression in class I (Figures 2E and 2F), class II, and class III neurons. These distinct patterns for Balboa::GFP localization were unlikely to be a consequence of the C-terminal GFP tag disrupting proper protein distribution because an N-terminally tagged Balboa transgene (*UAS-Venus::balboa*) showed an identical distribution (Figure S2A). In addition, the uniform pattern observed in class IV neurons was also seen with very low expression levels of the transgene (Figure S2B), suggesting that the uniform pattern was not an abnormal consequence of a very high expression level.

We reasoned that the distinct subcellular localization for Balboa::GFP proteins in the different neuronal classes might be due to differential PPK expression among md neuron subtypes. *ppk* is expressed at high levels in the class IV neurons, but it is expressed at low or undetectable levels in the class I (Figure 1A), class II, and class III neurons. Indeed, we found that simultaneous coexpression of *ppk* and *balboa::GFP* resulted in a Balboa::GFP distribution that was essentially uniform in all the md neurons (Figures 3A–3C). These results were strikingly different from what we observed with Balboa::GFP alone (Figures 2A and 2B). In the absence of additional PPK, the Balboa::GFP foci appeared evenly spaced, like beads on

and *knot* mRNA in nociceptors. The panmultidendritic marker *multiple* is present in both class I and class IV neurons.

(B) *ppk26-GAL4 > UAS-mCD8::GFP* exclusively labels the class IV md neurons (ddaC neuron is shown; the scale bar represents 20 μ m).

(C) Within the CNS, *ppk26-GAL4 > UAS-mCD8::GFP* exclusively labels projections of class IV neurons and is absent from central neurons (third instar ventral nerve cord, dorsal view; the scale bar represents 20 μ m).

(D) Knockdown of *balboa* severely impairs nociceptive behavioral responses of larvae stimulated with a 30 mN Von Frey fiber. The proportion of larvae exhibiting nociception responses was 67% of control animals without a driver (*w/yv*; *CG8546^{JF01843}/+* [n = 67]), 59% of control animals with the driver alone (*w/yv;ppk-GAL4/+; UAS-dicer-2/attp2* [n = 64]), and 21% of animals with *balboa*-RNAi targeted in class IV md neurons (*w;ppk-GAL4/+; UAS-dicer-2/CG8546^{JF01843}* [n = 62]). ***p < 0.001, Fisher's exact test with Bonferroni correction.

(E) Knockdown of *balboa* did not affect ChR2-triggered nociception behavior. Optogenetic activation of nociception behavior was seen in 84% of control animals (*w;ppk-GAL4 UAS-ChR2::eYFP Line C/+; UAS-dicer-2/+* [n = 111]). Knockdown of *balboa* did not significantly reduce the frequency of nociception responses to blue light (*w;ppk-GAL4 UAS-ChR2::eYFP Line C/+; UAS-dicer-2/CG8546^{GD2350}* [n = 113, mean = 78%]). p = 0.31, Fisher's exact test.

Error bars in (A) denote SEM. Error bars in (D) and (E) denote SE of the proportion.

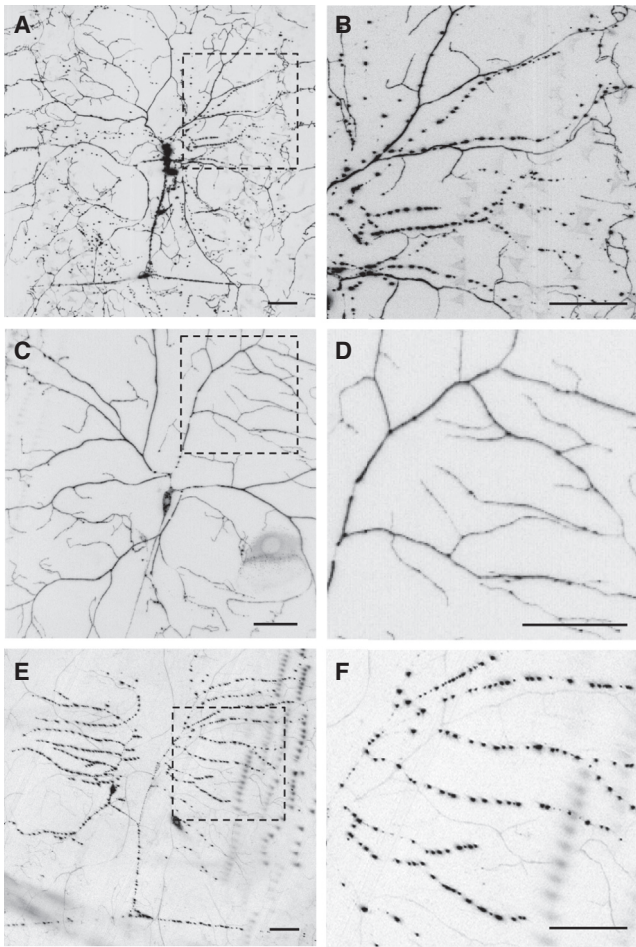


Figure 2. The Subcellular Localization of Balboa::GFP Varies among Multidendritic Neuron Subtypes

(A–F) The subcellular distribution of Balboa::GFP in the dorsal cluster of multidendritic (md) neurons. Expression of Balboa::GFP under the control of *md-GAL4* (A and B), *ppk-GAL4* (C and D), and *2-21-GAL4* (E and F) shows a broad, diffuse localization pattern throughout the arbor of class IV ddaC neurons but a punctate expression pattern in the dendrites and axons of class I, class II, and class III neurons. High magnification images (B, D, and F) of insets of (A), (C), and (E), respectively. Scale bars represent 50 μm . Genotypes shown are *w; md-GAL4/+; UAS-balboa::GFP/+* (A and B), *w; ppk-GAL4/+; UAS-balboa::GFP/+* (C and D), and *w; 2-21-GAL4/UAS-balboa::GFP* (E and F).

In these and all subsequent confocal micrographs, anterior is to the left and dorsal is at the top, and all images are of the dorsal, peripheral md neuron cluster of third instar larvae.

a string (Figures 3D–3F). This dramatic change in localization was particularly evident when we specifically examined the pattern within the class I neurons using the *2-21-GAL4* driver (Figures 3G–3K). In the presence of PPK, this Balboa::GFP localization pattern was converted to an even and uniform one where the fluorescence evenly filled the dendrites (Figures 3G–3I and 3K).

If coexpression of PPK and Balboa are indeed necessary for each other's stability and/or localization, then removing PPK or Balboa from the class IV neurons should alter the distribution of the cognate partner protein. Indeed, when we tested this prediction through knockdown of *ppk* in the class IV neurons, the normally uniform distribution of Balboa::GFP (Figures 4A and 4B) was converted to a clearly punctate distribution

(Figures 4C and 4D). Thus, reducing *ppk* expression caused a mislocalization of the Balboa::GFP protein to punctae within the class IV neurons.

In converse experiments, we generated transgenic animals for expression of fluorescently tagged Venus::PPK protein (*UAS-Venus::ppk*). As with tagged Balboa proteins, Venus::PPK distributed uniformly in the dendrites of class IV neurons (Figure 4E). We then used this transgene to test whether PPK localization was Balboa dependent via RNAi knockdown of *balboa* in the class IV neurons. This manipulation caused a dramatic reduction in the expression levels of Venus::PPK, to the point that it became undetectable in class IV neurons (Figure 4F). Importantly, *balboa-RNAi* did not reduce expression of *ppk* mRNA (Figure S1B) or the strength of *ppk-GAL4* expression (Figures S1C and S1D), indicating that these effects on Venus::PPK were not at the level of transcription or mRNA stability.

The above observations provided strong suggestive evidence that PPK protein subunits and Balboa protein subunits may physically interact *in vivo* to form a functional DEG/ENaC ion channel. Cells in which Balboa and PPK were coexpressed showed a uniform distribution of Balboa::GFP, whereas cells that expressed only Balboa::GFP displayed the punctate or aggregated distribution pattern. In addition, the expression of Venus::PPK was also dependent on the presence of Balboa. Nevertheless, it remained possible that these mutually dependent effects were indirect. For example, expression of PPK and/or Balboa within neurons might have regulated another protein (or proteins) that then in turn caused changes in the distribution of the cognate subunits.

Thus, to test for a direct physical interaction between Balboa protein subunits and PPK protein subunits, we utilized the split-GFP technique [14, 15]. In this approach, GFP is split into an N-terminal fragment (NGFP) and a C-terminal fragment (CGFP). The cleavage is performed in such a way that neither GFP protein fragment is able to form a radiative fluorophore upon cellular coexpression. However, when the NGFP and the CGFP fragments are coexpressed as fusions to other proteins that physically interact, the two GFP fragments come into sufficiently close proximity to permit reconstitution of GFP fluorescence [14].

To apply this approach to Balboa and PPK, we generated flies containing UAS transgenes that encoded a *balboa::CGFP* transcript, an *NGFP::balboa* transcript, or an *NGFP::ppk* transcript. As expected, neither the *balboa::CGFP* transcript nor the *NGFP::ppk* transcript produced detectable GFP fluorescence when individually expressed in neurons (data not shown). However, coexpression of the two transcripts together in all four classes of md neurons (Figure 4G) or specifically in class IV neurons (data not shown) caused clear reconstitution of GFP in the dendrites, soma, and axon. These results are consistent with a model in which the PPK-dependent redistribution of Balboa::GFP observed above is due to direct physical interactions between the proteins.

We next used the split-GFP protein approach to further test for homophilic *in vivo* interactions among Balboa subunits. Remarkably, upon coexpression of *balboa::CGFP* and *NGFP::balboa* in all four classes of md neurons, we observed GFP reconstitution that was restricted to class IV md neurons (Figure 4H). In surprising contrast, coexpression of *balboa::CGFP* and *NGFP::balboa* did not result in detectable GFP reconstitution in the class I, class II, or class III md neuron cell types. These findings further suggest that homophilic interactions among Balboa subunits do occur, but these interactions are

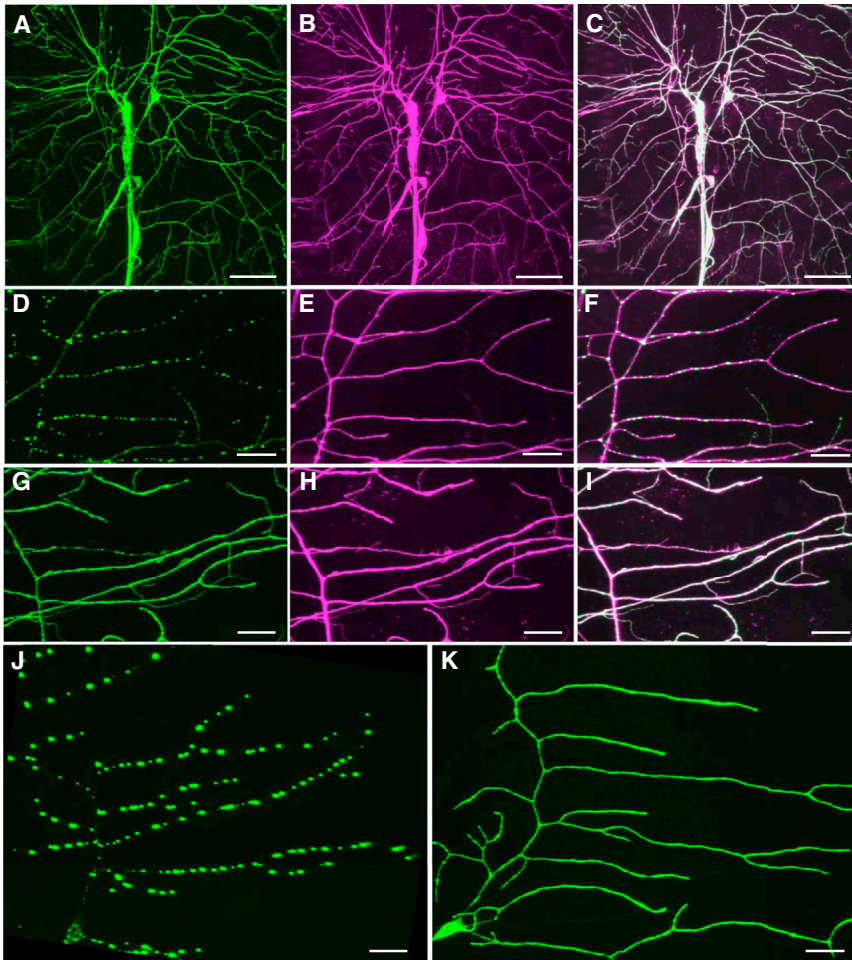


Figure 3. Ectopic Expression of PPK Alters the Subcellular Localization of Balboa::GFP

(A–C) Balboa::GFP was uniformly distributed throughout the dendritic arbors of all four classes of md neuron when PPK was ectopically coexpressed under the control of the *md-GAL4* driver (*w; md-GAL4 UAS-mCD8::DsRed /+; UAS-balboa::GFP/UAS-ppk*). (A) Balboa::GFP channel. (B) Counterlabel of the neuronal plasma membrane (mCD8::DsRed). (C) Merge of (A) and (B). (D–F) Expression of Balboa::GFP alone is punctate in class I and class II neurons, although dendrites show normal morphology (*w; md-GAL4 UAS-mCD8::DsRed /+; UAS-balboa::GFP/+*). (D) Balboa::GFP. (E) mCD8::DsRed. (F) Merge of (D) and (E). (G–I) Coexpression of PPK in class I and class II shows uniformly localized Balboa::GFP expression (*w; md-GAL4 UAS-mCD8::DsRed /+; UAS-balboa::GFP/UAS-ppk*). (G) Balboa::GFP. (H) mCD8::DsRed. (I) Merge of (G) and (H). (J) Ectopically expressed Balboa::GFP in class I neurons shows punctate expression under the control of the *2-21-GAL4* driver (*w; UAS-balboa::GFP/+; 2-21-GAL4/+*). (K) Balboa::GFP with coexpressed PPK under control of *2-21-GAL4* shows broad distribution in dendrites (*w; UAS-balboa::GFP/+; 2-21-GAL4/UAS-ppk*). Scale bars of (A)–(C) represent 50 μm ; scale bars of (D)–(K) represent 20 μm .

confined to the class IV neurons that also express PPK. Combined with the findings that Balboa::GFP distribution in class IV neurons also depends on PPK, these results suggest that homophilic Balboa interactions are likely to also depend on the presence of PPK. Interestingly, the Balboa::GFP punctate structures seen in class I, class II, and class III neurons are not observed in experiments with reconstituted GFP. This may be due to a relatively long maturation time for the split-GFP fluorophore [16] and might indicate that Balboa::GFP present in punctae is less stable relative to the uniformly distributed Balboa::GFP. Alternatively, Balboa proteins in punctae do not form homophilic interactions.

In addition to these *in vivo* experiments, we used the split-GFP approach to further investigate interactions among Balboa and PPK subunits when transiently expressed in S2R+ cells (Figures S3A–S3F). As in md neurons, *in vitro* reconstitution of GFP was detected at the plasma membrane with heteromeric channels (NGFP::PPK + Balboa::CGFP, Figure S3B). Interestingly, homophilic interactions among PPK subunits (Figure S3C) or Balboa subunits (Figure S3E) were confined to intracellular secretory structures in the absence of the heteromeric partner. In contrast, the homophilic interactions were clearly detectable at the plasma membrane upon cotransfection with the untagged heteromeric partner (Figures S3D and S3F). Combined, these data suggest that plasma membrane localization of Balboa and PPK each depend on the presence of the heteromeric partner protein. Furthermore, as both homophilic Balboa

and PPK interactions were detected at the plasma membrane, our results suggest that both types of heterotrimeric channels (i.e., either BBA/PPK/BBA or PPK/BBA/PPK) may be formed in S2R+ cells.

Are Balboa and PPK heteromeric channels sufficient to form a mechanosensitive channel? To test for this possibility, we coexpressed the subunits heterologously both in HEK293t cells and in *Drosophila* S2R+ cells (Figures S4A–S4D) and tested for mechanosensitive currents. Whereas the known mechanosensitive channel MPiezo1 and DmPiezo2 produced robust currents when stimulated either with a blunt glass pipette (poke) or with negative pressure (stretch) (Figures S4A–S4C), cells transfected with *balboa*, *ppk*, or with *balboa* and *ppk* together did not exhibit currents above background. Lack of currents from coexpression of *ppk* and *balboa* may be explained by the need for other accessory proteins for proper function. This is exemplified by observations indicating that the MEC-2 stomatin protein dramatically increased the currents generated from mutant versions of MEC-4 and MEC-10 in *Xenopus* oocytes [17]. Similarly, mammalian stomatin domain proteins also regulate acid sensing ion channels in the DEG/ENaC family [18].

In summary, our study again demonstrates the power of forward genetics in the identification of novel components important in nociception signaling pathways. Converging lines of evidence strongly support the hypothesis that Balboa and PPK physically interact and likely form a functional ion channel

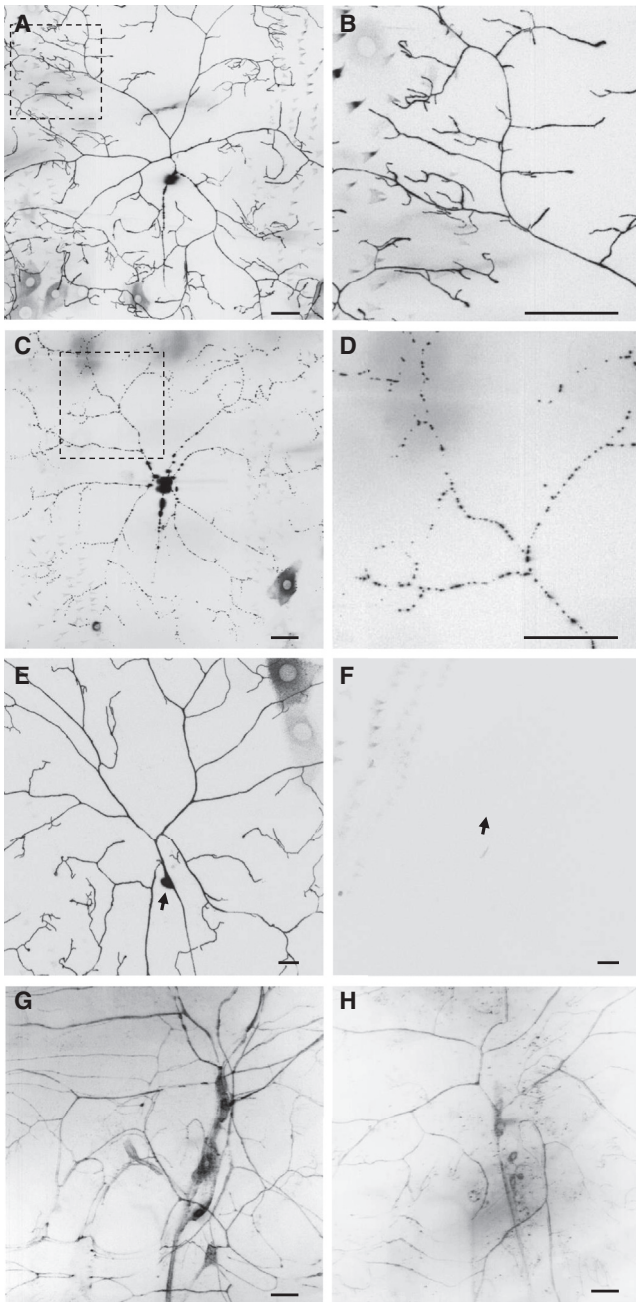


Figure 4. Balboa and PPK Physically Interact In Vivo

(A) Balboa::GFP shows a broad distribution pattern in dendrites and axons of the class IV ddaC neuron (boxed area shown in more detail in B). (B) Zoomed-in view of the boxed area in (A). (C) Balboa::GFP is distributed in discrete bright foci in *ppk*-RNAi animals (boxed area is shown in more detail in D). (D) Zoomed-in view of the boxed area in (C). (E) Venus::PPK is distributed uniformly in the dendrites of class IV neurons (arrow indicates cell body). (F) *balboa*-RNAi eliminates Venus::PPK from class IV neurons (arrow indicates the location of the cell body). (G) GFP is reconstituted in the dendrites of class I–IV neurons when NGFP::PPK and Balboa::CGFP are coexpressed. (H) When Balboa::CGFP and NGFP::Balboa are coexpressed in class I–IV neurons, reconstitution of GFP is limited to the class IV neurons. Scale bars represent 20 μ m. Genotypes shown are *w*; *ppk-GAL4/+*; *UAS-dicer-2/UAS-balboa::GFP/+* (A and B), *w*; *ppk-GAL4/ppk^{KK104185}*; *UAS-dicer-2/UAS-balboa::GFP* (C and D), *w*; *ppk-GAL4/+*; *UAS-dicer-2/*

in vivo. First, both genes are functionally required for mechanical nociception. Second, both genes show very specific enrichment and expression in the class IV nociceptor neurons. Third, *ppk* expression causes a dramatic redistribution of Balboa::GFP fluorescence in the md neurons. Fourth, the Balboa::GFP distribution is altered by *ppk* knockdown, and Venus::PPK is dramatically reduced by *balboa* knockdown. Fifth, GFP is reconstituted by coexpression of *balboa::CGFP* and *NGFP::ppk* within neurons of the larval peripheral nervous system.

It is noteworthy that although many DEG/ENaCs have been shown to form homotrimeric or heterotrimeric channels [19, 20], our findings using the split-GFP approach provide the first direct measurement of such physical interactions between heteromeric DEG/ENaC subunits *in vivo*. Interestingly, GFP reconstitution measurements of homophilic Balboa interactions were confined to the class IV neurons. Thus, it is highly likely that these *in vivo* homophilic interactions depend on the presence of PPK. Indeed, results in S2R+ cells suggest that the presence of PPK is needed for plasma membrane localization of Balboa.

A surprising aspect of our findings is that Balboa::GFP, Venus::Balboa, and Venus::PPK show uniform labeling throughout the dendritic arbor of the nociceptive class IV neurons. This pattern differs from the previously described punctate localization pattern of MEC-4/MEC-10 in *C. elegans* mechanosensory neurons. Indeed, our results suggest that the uniform dendritic localization pattern for the Balboa::GFP protein is the functionally relevant pattern for mechanical nociception. Perhaps, the uniform pattern might contribute to the high mechanosensory threshold of these cells. Further experimentation will be needed to identify the structures marked by the ectopic punctate pattern seen in class I, class II, and class III neurons. Because Balboa localization to the plasma membrane depends on the presence of PPK, these structures likely represent an unknown component of the secretory pathway or sites for protein degradation. To our knowledge, similarly labeled intracellular structures in md neurons have not been previously observed. Future analyses of these structures may thus provide important insight into the regulation of mechanosensory ion channel biosynthesis or turnover.

Experimental Procedures

For detailed experimental procedures, see [Supplemental Experimental Procedures](#).

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at <http://dx.doi.org/10.1016/j.cub.2014.10.038>.

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UAS-Venus::PPK (E), *w*; *ppk-GAL4/UAS-Venus::PPK*; *UAS-dicer-2/UAS-balboa-RNAi^{JF01843}* (F), *w*; *md-GAL4/+*; *UAS-NGFP::ppk/UAS-balboa::CGFP* (G), and *w*; *md-GAL4/+*; *UAS-balboa::CGFP/UAS-NGFP::Balboa* (H).

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