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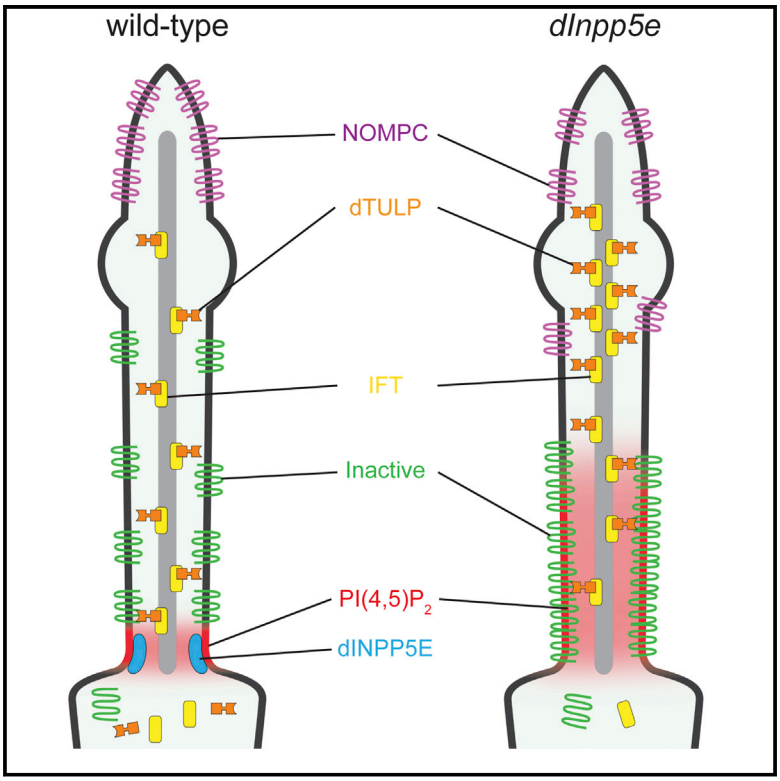
Ciliary Phosphoinositide Regulates Ciliary Protein Trafficking in *Drosophila*

Graphical Abstract



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In Brief

Park et al. report that *Drosophila* INPP5E regulates PI(4,5)P_2 levels in the ciliary membrane. Loss of *dInpp5e* increases PI(4,5)P_2 levels in the ciliary base. This, in turn, causes a ciliary accumulation of dTULP and consequent mislocalization of IAV and NOMPC.

Highlights

- Deletion of *dInpp5e* increases ciliary localization of dTULP
- Deletion of *dInpp5e* increases PI(4,5)P_2 levels in the ciliary base
- Augmenting PI(4,5)P_2 levels disturbs the ciliary distribution of TRP channels
- dTULP detection of PI(4,5)P_2 affects ciliary TRP channel trafficking

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Ciliary Phosphoinositide Regulates Ciliary Protein Trafficking in *Drosophila*

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SUMMARY

Cilia are highly specialized antennae-like cellular organelles. Inositol polyphosphate 5-phosphatase E (INPP5E) converts PI(4,5)P_2 into PI4P and is required for proper ciliary function. Although *Inpp5e* mutations are associated with ciliopathies in humans and mice, the precise molecular role INPP5E plays in cilia remains unclear. Here, we report that *Drosophila* INPP5E (dINPP5E) regulates ciliary protein trafficking by controlling the phosphoinositide composition of ciliary membranes. Mutations in *dInpp5e* lead to hearing deficits due to the mislocalization of dTULP and mechanotransduction channels, Inactive and NOMPC, in chordotonal cilia. Both loss of dINPP5E and ectopic expression of the phosphatidylinositol-4-phosphate 5-kinase Skittles increase PI(4,5)P_2 levels in the ciliary base. The fact that Skittles expression phenocopies the *dInpp5e* mutants confirms a central role for PI(4,5)P_2 in the regulation of dTULP, Inactive, and NOMPC localization. These data suggest that the spatial localization and levels of PI(4,5)P_2 in ciliary membranes are important regulators of ciliary trafficking and function.

INTRODUCTION

Cilia are specialized organelles that extend from the surface of many cell types. Cilia are essential for processes as diverse as developmental signaling and adult homeostasis (Drummond, 2012; Fliegauf et al., 2007). Ciliary dysfunction leads to a range of diseases called ciliopathies. Examples include polycystic kidney disease, retinitis pigmentosa, Bardet-Biedl syndrome, and Joubert syndrome (Green et al., 1989; Liu et al., 2002; Pazour et al., 2000; Valente et al., 2006).

Although the ciliary membrane is continuous with the plasma membrane, lateral diffusion of membrane components is so limited that the ciliary membrane represents a distinct membrane compartment (Chih et al., 2012; Hu et al., 2010; Nachury et al., 2010). The ciliary membrane holds distinct populations of transmembrane signaling molecules, including platelet-derived growth factor receptor, Smoothened, transient receptor potential (TRP) channels, and G-protein-coupled receptors that participate in both sensory transduction and developmental signaling (Colbert et al., 1997; Corbit et al., 2005; Ezratty et al., 2011; Rohatgi et al., 2007). An elaborate gating mechanism in the transition zone of the ciliary base establishes and maintains the compartmentalization that is necessary for proper ciliary function—it regulates the entry, localization, and exit of specific signaling molecules to and from the ciliary compartment (Chih et al., 2012; Williams et al., 2011).

Intraflagellar transport (IFT) is a specialized form of protein trafficking required not only for the formation and maintenance of cilia themselves but also for the trafficking of transmembrane receptors required for ciliary function through the diffusion barrier found at the ciliary base (Crouse et al., 2014; Keady et al., 2011; Nachury et al., 2010). IFT is primarily accomplished via multi-protein particles known as IFT-A and IFT-B (Rosenbaum and Witman, 2002). Some protein cargoes, however, have been reported to enter cilia independent of the IFT pathway (Bellzile et al., 2013). In addition to the proteins classified as IFT components, several other proteins participate in the targeting of transmembrane proteins into the cilia compartment. These include the components of the BBSome, as well as Arf GTPase, Rab GTPase, and tubby-like protein 3 (TULP3) (Berbari et al., 2008; Crouse et al., 2014; Deretic et al., 2005; Keady et al., 2011; Mukhopadhyay et al., 2010; Nachury et al., 2007; Wang et al., 2012). Although many of the players have been identified, the molecular mechanisms that permit the remarkable sorting specificity of protein cargoes into the ciliary compartment are still not fully understood.

We and others have previously shown that dTULP, the *Drosophila* tubby homolog, and TULP3, a member of the mammalian tubby-like protein family, regulate ciliary trafficking of membrane receptors in fruit flies and mammals, respectively (Mukhopadhyay et al., 2010; Park et al., 2013). dTULP and TULP3 share the ability to bind to IFT components and to phosphoinositides (PIPs), and both of these properties are required for receptor trafficking to cilia. It is, therefore, tempting to speculate that PIPs may represent another important regulator of ciliary protein trafficking, possibly in cooperation with IFT. Indeed, several recent reports suggest PIPs are critical in normal ciliary function. Mutation of 5-phosphatase, *cil-1* in *C. elegans,* causes mislocalization of the ciliary receptors PKD-2 and LOV-1 (Bae

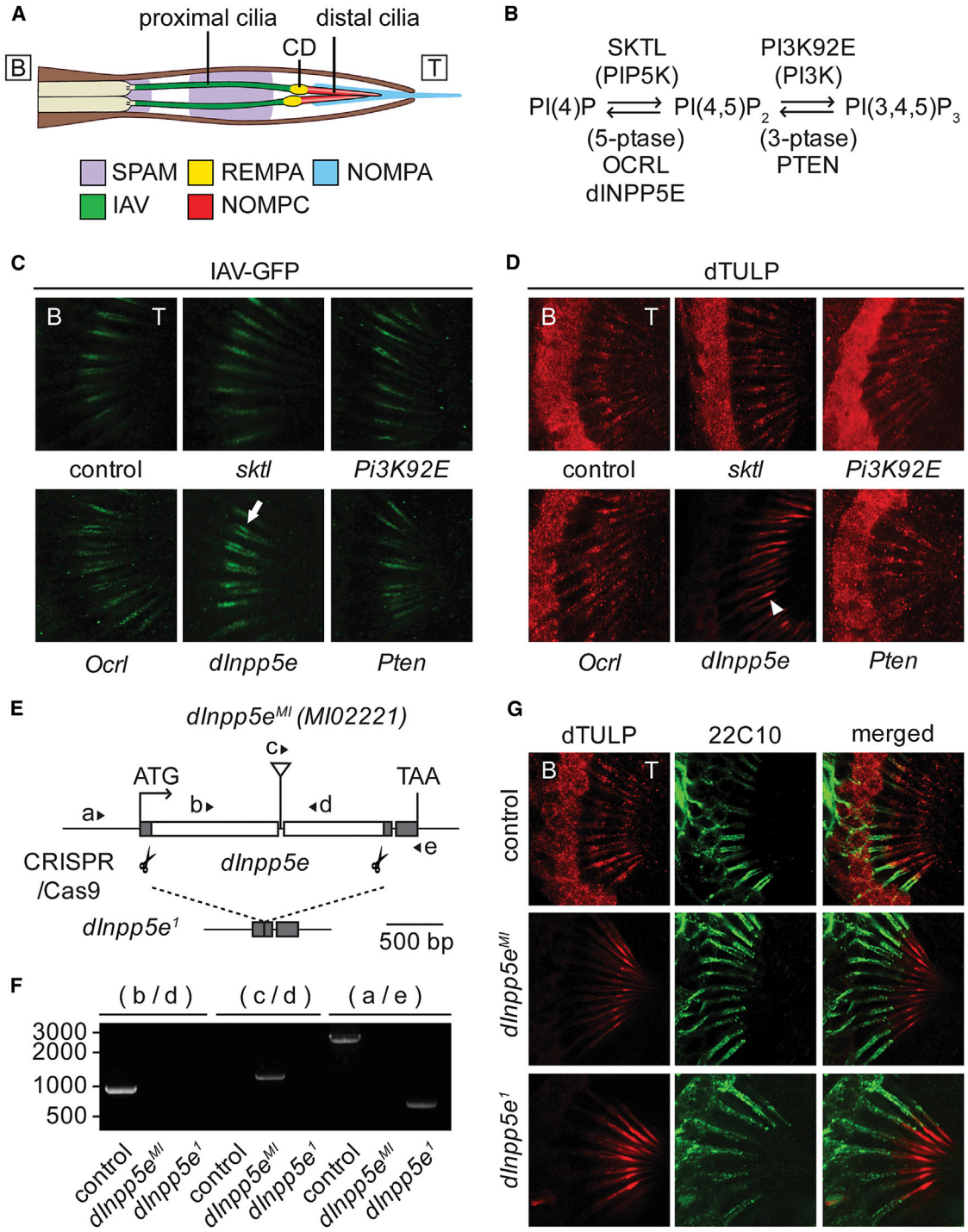

Figure 1. Modulation of Phosphoinositide Signaling Alters Protein Localization in Chordotonal Neuron Cilia

(A) Schematic showing chordotonal neuron structure. Molecular localization patterns are indicated in various colors. The locations of the cell body (B), ciliary dilation (CD), and ciliary tip (T) are indicated.

(B) The phosphoinositide (PIP) signaling cascade. The kinases and phosphatases that mediate each lipid conversion in *Drosophila* are depicted. PIP5K, phosphatidylinositol-4-phosphate 5-kinase, SKTL, skittles; PI3K, phosphatidylinositol 3-kinase; 5-ptase, phosphatidylinositol 4,5-bisphosphate 5-phosphatase; OCRL, Oculocerebrorenal syndrome of Lowe; 3-ptase, phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase; PTEN, phosphatase and tensin.

(C) RNAi screen of PIP kinases and phosphatases for impairments in IAV localization. RNAi lines were crossed to the *UAS-Dcr2*;*elav-GAL4*,*Iav-GFP* driver line. The IAV-GFP signal (green) was visualized by immunostaining with GFP antibody. The arrow indicates the enrichment of the IAV signal in proximal cilia. Orientation of the chordotonal cilia is indicated using the letters B (cell body) and T (tip).

(legend continued on next page)

et al., 2009). Disruption of the enzymatic activity or the ciliary targeting of INPP5E leads, respectively, to the ciliopathies Joubert syndrome and MORM (mental retardation, truncal obesity, retinal dystrophy, and micropenis) syndrome (Bielas et al., 2009; Jacoby et al., 2009). Despite this progress, detailed information regarding the altered distribution of specific PIPs and their resulting molecular and physiological effects on ciliary function is still lacking.

Here, we show that *Drosophila* INPP5E (hereafter dINPP5E) regulates membrane protein trafficking in chordotonal sensory cilia. Inactivation of dINPP5E increases the levels of phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) in the ciliary base and leads to ciliary accumulation of dTULP and the subsequent mislocalization of its cargos Inactive (IAV) and NOMPC. Elimination of dTULP's PIP-binding ability partially rescues the reduced sound-evoked potentials and mislocalization of IAV and NOMPC of *dInpp5e* mutants. Our data, thus, support a role for PIPs in the ciliary membrane as direct regulators of dTULP, and therefore indirect regulators of the trafficking of the mechanotransduction channels IAV and NOMPC.

RESULTS

dInpp5e Knockdown Changes Ciliary Localization of IAV and dTULP

Previously, we demonstrated that dTULP, the *Drosophila* Tubby homolog, regulates the ciliary localization of IAV and NOMPC—TRP channels that are essential for hearing in *Drosophila* and are expressed in the antennal chordotonal neurons (Park et al., 2013). The outer dendritic segments of the chordotonal neurons are compartmentalized cilia divided into structurally distinct proximal and distal sections (Figure 1A). IAV is a component of the hearing transduction complex and is localized in the proximal cilia, while NOMPC modulates hearing from its location in the distal cilia (Eberl et al., 2000; Gong et al., 2004; Lee et al., 2010; Lehnert et al., 2013). dTULP is required for IAV to enter the cilia and is also necessary for the distal ciliary localization of NOMPC (NOMPC ciliary entry is independent of dTULP). Mutations in the dTULP PIP-binding domain reduce the localization of IAV to the proximal cilia of chordotonal neurons. This suggests PIPs may be important regulators in ciliary protein trafficking.

Given that the conserved C-terminal PIP-binding domain of the tubby-like protein family members shows a higher binding affinity for $PI(4,5)P_2$ over other PIPs (Mukhopadhyay et al., 2010; Santagata et al., 2001), we focused on five PIP-modifying enzymes in flies that are known to directly regulate $PI(4,5)P_2$ levels (Figure 1B). These include the PIP5 Kinase Skittles, the PI3 Kinase PI3K92E, the 5-phophatases OCRL and CG10426 (dINPP5E), and the 3-phosphatase PTEN. We, therefore, crossed flies carrying *UAS-RNAi* transgenes specific to these PIP-modifying enzymes to flies carrying the pan-neuronal *elav-GAL4* driver transgene and a *UAS-Dcr2* transgene to enhance RNAi efficiency. Although all the enzymes we chose to knock down should theoretically alter $PI(4,5)P_2$ levels, we looked specifically for those that significantly affected ciliary protein trafficking in chordotonal neurons.

Of the five PIP-modifying enzymes tested, knockdown of *CG10426*, which we name here *dInpp5e,* gives the most dramatic phenotype. It leads to the accumulation and uneven distribution of IAV at the ciliary base of chordotonal neurons rather than the proximal cilia as in controls (Figure 1C). *dInpp5e* encodes a 747 amino acid-long phosphatase that is 43% identical to human INPP5E (Figure S1A). We also visualized the localization of dTULP upon knockdown of the same five PIP-modifying enzymes. Surprisingly, *dInpp5e* knockdown leads to significant ciliary accumulation of dTULP with a corresponding reduction in the level of dTULP in the chordotonal neuron cell bodies. Knockdown of other PIP-modifying enzymes, however, does not affect dTULP localization (Figure 1D).

dInpp5e Loss of Function Impairs dTULP, IAV, and NOMPC Localization

To confirm the role of dINPP5E in the regulation of IAV and dTULP localization, we obtained a Minos element insertion in a *dInpp5e* intron ($dInpp5e^{MI}$) (Figure 1E). We also generated another mutant strain, $dInpp5e^{1}$, using the clustered regularly interspaced short palindromic repeat-associated nuclease 9 (CRISPR/Cas9) technique (Gratz et al., 2013). $dInpp5e^{1}$ lacks the portions of *dInpp5e* coding exons that code for amino acids 39 through 661 of dINPP5E, more than 80% of the final protein (Figure 1E). We confirmed the location of the Minos element insertion between exons in $dInpp5e^{MI}$ flies and the location of the genomic deletion of $dInpp5e^{1}$ by genomic PCR (Figure 1F). Both mutant alleles are homozygous viable and fertile. Consistent with our RNAi results, both *dInpp5e* mutant strains showed abnormal dTULP accumulation inside chordotonal cilia accompanied by a dramatic decrease in cell body staining (Figure 1G). This change in ciliary dTULP accumulation in the *dInpp5e* mutants cannot be attributed to an increase in dTULP protein levels, because dTULP protein levels in the *dInpp5e* mutant antennae are not different from that in control antennae (Figure S1B).

Next, we examined the localization of other ciliary membrane proteins in *dInpp5e* mutant flies. Also consistent with our RNAi results from Figure 1, IAV is enriched in the ciliary base of *dInpp5e* mutant flies compared to controls (Figure 2A). Although NOMPC is normally found in the distal cilia of chordotonal

(D) RNAi screen of PIP kinases and phosphatases for impairments in dTULP localization. RNAi lines were crossed to the *elav-GAL4,UAS-Dcr2* driver line and immunostained with dTULP antibody (red). The arrowhead indicates the ciliary dilation.

(E) A map of the *dInpp5e* locus indicating the molecular nature of the $dInpp5e^{MI}$ and $dInpp5e^{1}$ mutant alleles. The open inverted triangle indicates the location of the Minos insertion MI02221 ($dInpp5e^{MI}$). The $dInpp5e^{1}$ allele was generated using the CRISPR/Cas9 technique. Locations of the RNA-guided target sites are depicted with scissors. Gray boxes are coding exons that remain after the deletion. Filled arrowheads indicate the primers used for mutant verification.

(F) Genomic PCR confirming the insertion of the Minos element and the deletion of *dInpp5e* using primer pair indicated in Figure 1E.

(G) Localization of dTULP in the *dInpp5e* mutants. Immunostaining of dTULP counterstained with 22C10, which marks all neurons except for outer ciliary segments.

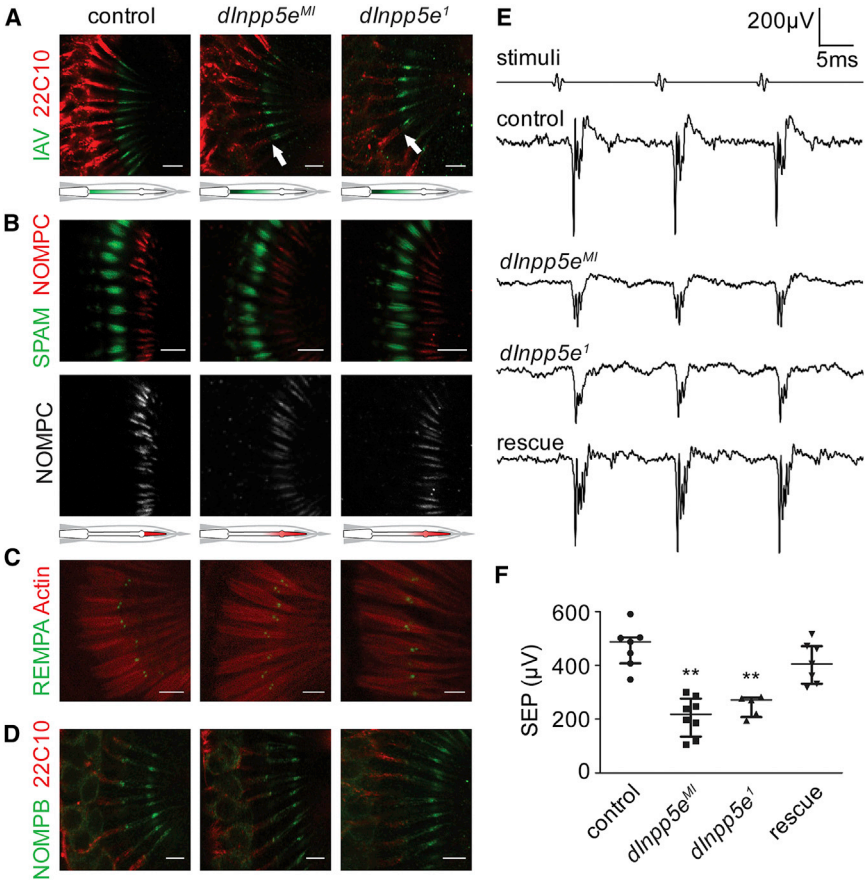

Figure 2. *dInpp5e* Loss of Function Causes Mislocalization of Ciliary Proteins and Reduces Sound-Evoked Potentials

(A) IAV localization in *dInpp5e* mutants. IAV-GFP (green) counterstained with 22C10 (red). Arrows indicate the junction between the inner segment and outer ciliary segments. Schematic shows IAV localization.

(B) Immunostaining of NOMPC and SPAM in *dInpp5e* mutants. Schematic shows NOMPC localization.

(C) REMPA localization in *dInpp5e* mutants. Immunostaining of REMPA-YFP (anti-GFP) counterstained with phalloidin, which specifically stains the actin-rich scolopales.

(D) NOMPB localization in *dInpp5e* mutants. Immunostaining of NOMPB-GFP (anti-GFP) counterstained with 22C10.

(E) Representative traces of sound-evoked potentials recorded from the antennal nerves from flies of the indicated genotypes. Rescue flies indicate a BAC-derived genomic rescue transgene introduced into the *dInpp5e*^{MI} background.

(F) Quantification of sound-evoked potentials. The medians and quartiles are indicated by horizontal lines. *p* values were calculated using the Kruskal-Wallis test and Mann-Whitney *U* post hoc tests (**p < 0.01).

All scale bars represent 50 μm.

neurons, it is reduced and slightly mislocalized toward the proximal cilia in *dInpp5e* mutants (Figure 2B). Spacemaker (Spam) is an extracellular protein that protects cilia from osmotic stress (Cook et al., 2008). It is typically localized in the luminal space of the scolopale, with one large accumulation in the proximal cilia close to the ciliary dilation and another small accumulation close to the ciliary base. Mutations in most IFT-A components and in *dTulp* are known to cause a mislocalization of Spam (Lee et al., 2010; Park et al., 2013). Interestingly, Spam localization is unaltered in *dInpp5e* mutants (Figure 2B). Localization of RempA/IFT140 and NOMPB/IFT88, components of the IFT-A and IFT-B complexes, respectively, are similarly unaltered in *dInpp5e* mutant antennae (Figures 2C and 2D). This suggests dINPP5E regulates the trafficking of only a subset of ciliary proteins.

dInpp5e Loss of Function Impairs Sound-Evoked Potentials

Previously, we showed that mislocalization of NOMPC and IAV disrupts hearing (Park et al., 2013). We, therefore, recorded extracellular sound-evoked potentials to determine whether the observed mislocalizations of ciliary proteins also disrupt the function of chordotonal neurons in the two *dInpp5e* mutant strains. The median amplitude of sound-evoked potentials is reduced from 466 μV in control flies (n = 7) to 217 μV in *dInpp5e*^{MI} (n = 8) and 271 μV in *dInpp5e*^{1} (n = 5) (Figures 2E and 2F). Concurrent expression of a wild-type *dInpp5e*$^{+}$ transgene rescues the reduction of sound-evoked potentials of *dInpp5e* mutants (*dInpp5e*^{MI}), suggesting their hearing defects are attributable to the mutation in *dInpp5e* (Figures 2E and 2F).

dInpp5e Loss of Function Increases PI(4,5)P_2 in the Ciliary Base

INPP5E is an enzyme that hydrolyzes either PI(3,4,5)P_3 to PI(3,4)P_2 or PI(4,5)P_2 to phosphatidylinositol 4-phosphate (PI4P) (Bielas et al., 2009). Although mutations in INPP5E are associated with ciliopathy, it is unclear which PIPs, if any, are present in ciliary membranes (Conduit et al., 2012). Thus, we examined whether dINPP5E loss of function increases PI(4,5)P_2 in the cilia of chordotonal neurons. Using PI(4,5)P_2-specific antibodies, we were able to detect PI(4,5)P_2 in the ciliary base of control chordotonal neurons (Figure S2A). Loss of dINPP5E leads to further accumulation of PI(4,5)P_2 in the ciliary base (Figure 3A). To verify the identity of this signal, we expressed in chordotonal cilia the *Drosophila* phosphatidylinositol- 4-phosphate 5-kinase Skittles, which converts PI4P to PI(4,5)P_2 (Hassan et al., 1998). We observed increases in the immunofluorescent signals in the same location when Skittles was expressed in cilia (Figure 3C). This suggests that dINPP5E hydrolyzes PI(4,5)P_2 to PI4P and prevents the accumulation of PI(4,5)P_2 in the ciliary base.

To determine the localization of dINPP5E, we examined chordotonal neurons expressing functional EGFP-tagged dINPP5E; this rescues the dTULP localization defect in *dInpp5e*^{1} flies (Figure S2B). The resulting EGFP-dINPP5E signal is limited to a

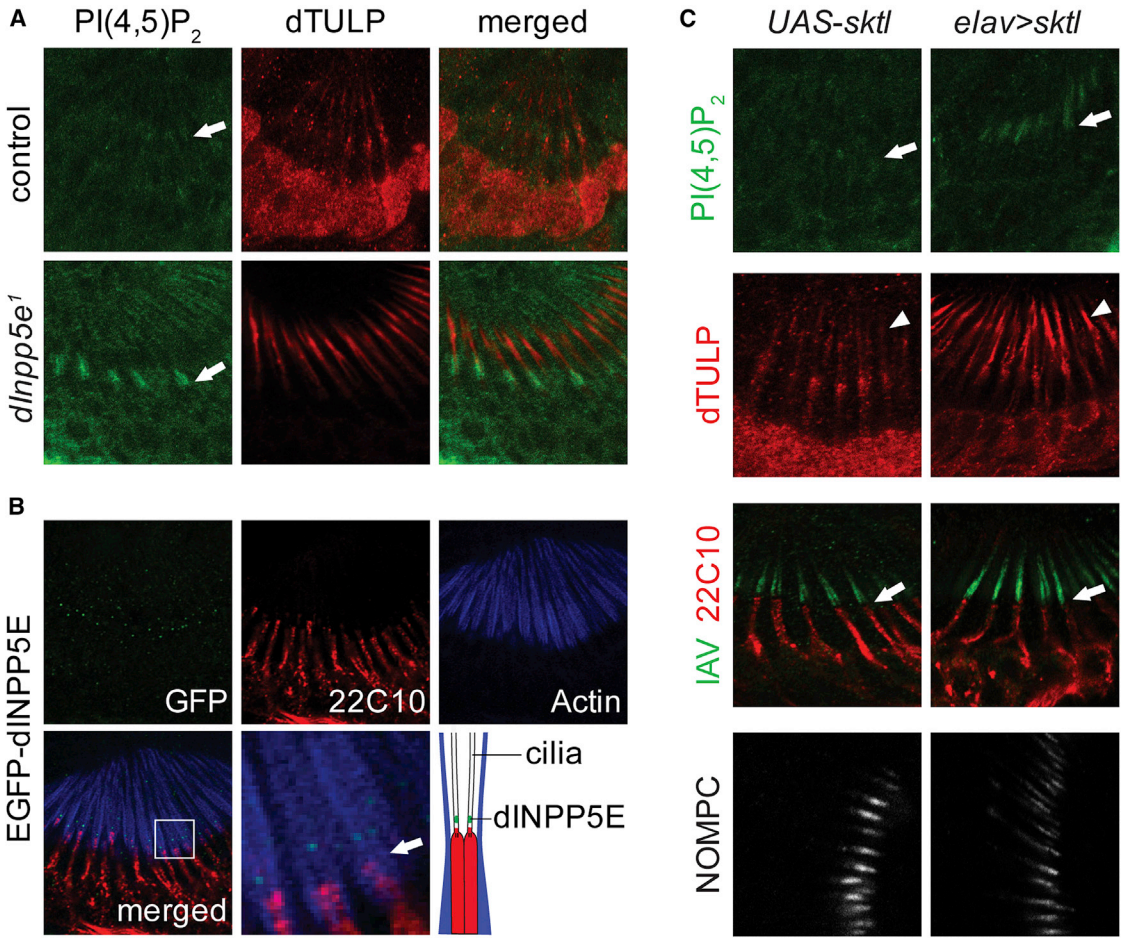

Figure 3. Elevated PI(4,5)P_2 in the Ciliary Base Alters Ciliary Protein Localization

(A) Level and localization of ciliary PI(4,5)P_2 induced by loss of dINPP5E. Immunostaining of PI(4,5)P_2 and dTULP in chordotonal neurons of the indicated genotypes. Arrows indicate the junction between the inner and outer ciliary segments.

(B) Subcellular dINPP5E localization. Immunostaining of EGFP-dINPP5E (anti-GFP, green) in chordotonal neurons counterstained with 22C10 (red) to show nonciliary structures and phalloidin to show the actin-rich scolopales (blue). The white box indicates the location of the higher magnification image to the right of the merged image. Arrows indicate the junction between the inner and outer ciliary segments. dINPP5E localization is also schematized in the lower right.

(C) Ectopic expression of the phosphatidylinositol-4-phosphate 5-kinase Sktl increases PI(4,5)P_2. Sktl was expressed in all neurons using the pan-neuronal *elav-GAL4* driver. Immunostaining of PI(4,5)P_2, dTULP, IAV-GFP (anti-GFP), and NOMPC are shown. Arrows indicate the junction between the inner segment and outer ciliary segments and arrowheads indicate the ciliary dilation.

single, well-defined focus in the ciliary base of chordotonal neurons, presumably in the transition zone (Figure 3B). In control flies, this dINPP5E staining is also surrounded by a region of relatively high concentration PI(4,5)P_2 (Figure S2C).

Artificial Elevation of PI(4,5)P_2 Mimics the *dInpp5e* Mutant Phenotype

The altered ciliary protein localization observed in the *dInpp5e* mutants may be caused by increases in PI(4,5)P_2 in the ciliary base, or it could instead be due to some other effects (e.g., an unknown scaffolding property of dINPP5E). To distinguish these possibilities, we ectopically expressed the phosphatidylinositol-4-phosphate 5-kinase Skittles as another mean of elevating PI(4,5)P_2 levels (Hassan et al., 1998). As expected, ectopic expression of Skittles increases PI(4,5)P_2 in ciliary base and causes dTULP accumulation in the chordotonal cilia (Figure 3C). In addition, elevated PI(4,5)P_2 also causes mislocalization of IAV and NOMPC in patterns similar to those of the *dInpp5e* mutants (Figure 3C). These results strongly suggest that changes in PI(4,5)P_2 levels account for these *dInpp5e* mutant phenotypes.

Mutation of dTULP's PIP-Binding Domain Partially Rescues *dInpp5e* Phenotypes

To further explore the relationship between PI(4,5)P_2 and dTULP trafficking and function, we investigated the effects of a simple mutation of the dTULP PIP-binding domain in dINPP5E loss-of-function mutants. Wild-type dTULP (dTULP^{wt}) and dTULP with a mutation in its PIP-binding domain (dTULP^{PIP-}) expressed in the *dTulp*^{1} mutant background using the chordotonal neuron driver *F-GAL4* (Kim et al., 2003) show similar levels of ciliary

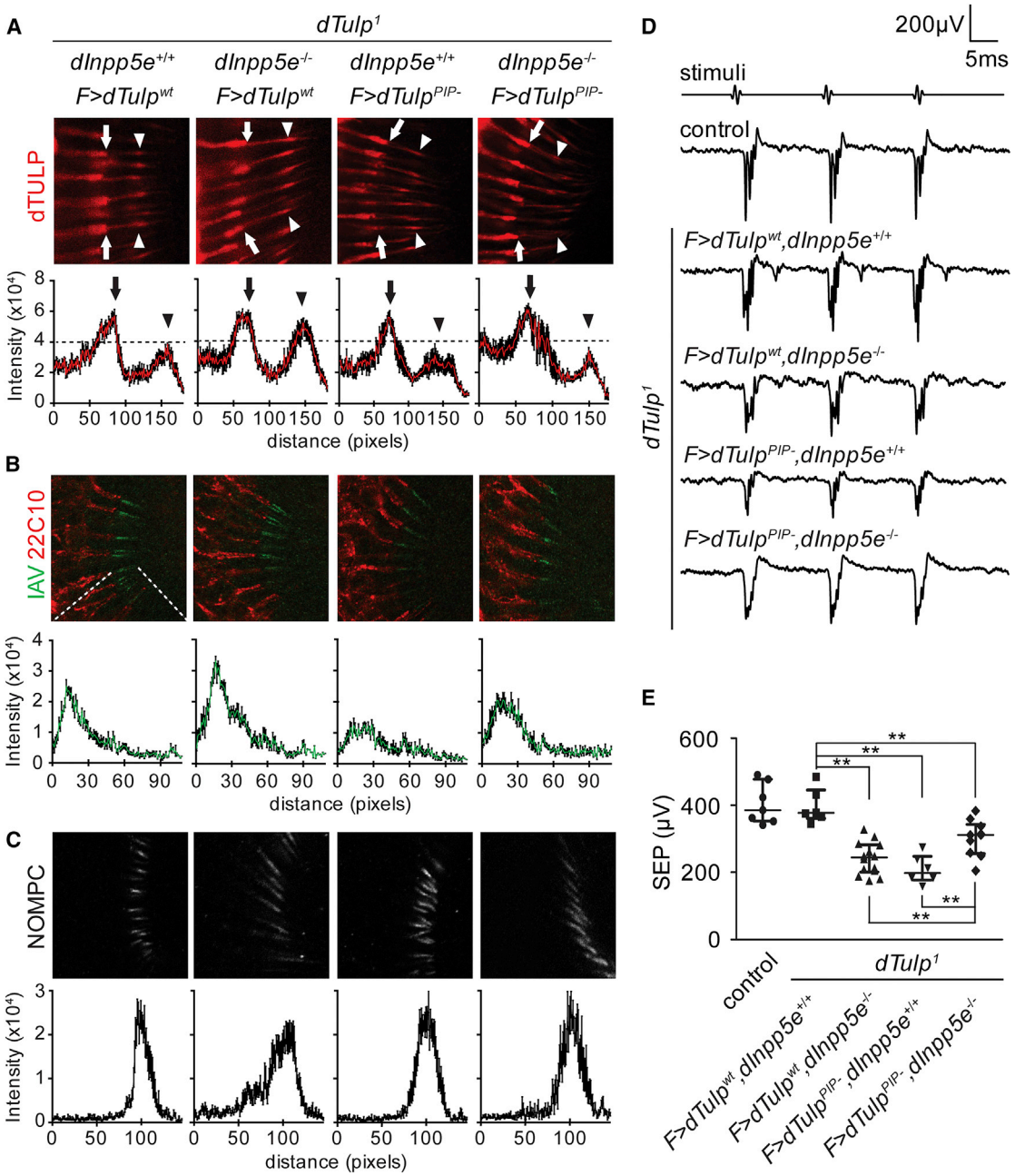

Figure 4. The dTULP PIP-Binding Domain Contributes to the Localization and Function of dTULP, IAV, and NOMPC

(A) (Top) Immunostaining of dTULP^{wt} or dTULP^{PIP-} expressed in the *dInpp5e*$^{+/+}$, *dTulp*^{1}, or *dInpp5e*$^{-/-}$ *dTulp*^{1} backgrounds. Arrows indicate the location of the junction between the inner and outer ciliary segments, and the arrowheads indicate the location of the ciliary dilation. (Bottom) Intensity profile of dTULP signal along the cilia. The dotted line indicates dTULP signal intensity at the ciliary dilation of the dTULP^{wt} rescue.

(B) (Top) Immunostaining of IAV-GFP in the same genotypes as in (A) along with a 22C10 counterstain for non-ciliary structures. (Bottom) Intensity profile of IAV-GFP signal along the cilia.

(C) (Top) Immunostaining of NOMPC in the same genotypes as in (A). (Bottom) Intensity profile of NOMPC signal along the cilia.

(D) Representative sound-evoked potentials from w^{1118} control flies or the same genotypes as in (A).

(E) Quantification of the sound-evoked potentials. The medians and quartiles are indicated by horizontal lines. *p* values were calculated using the Kruskal-Wallis test and Mann-Whitney *U* post hoc tests (**p < 0.01). *dInpp5e*$^{-/-}$ indicates the *dInpp5e*^{MI} strain.

accumulation (*FdTulp*^{wt};*dTulp*^{1} and *FdTulp*$^{PIP-}$;*dTulp*^{1}; Figure 4A). When expressed in the *dTulp*^{1} and *dInpp5e*$^{-/-}$ (*dInpp5e*^{MI}) double-mutant background, however, dTULP^{wt} showed significantly more ciliary enrichment around the ciliary dilation than dTULP^{PIP-} (Figure 4A). In *dInpp5e*$^{-/-}$, antennae expressing wild-type dTULP had slightly elevated levels of IAV. In

addition, the localization of both IAV and NOMPC is skewed toward the ciliary base. These phenotypes, however, are partially rescued by an alternative expression of the PIP-binding domain mutant dTULP^{PIP-} (Figures 4B and 4C). In addition to these changes in protein localization, *dINPP5E* loss of function and the mutation of the dTULP PIP-binding domain similarly reduce antennal sound-evoked potentials (Figures 4D and 4E). Surprisingly, the combination of the two (*FdTulp*$^{PIP-}$ and *dInpp5e*$^{-/-}$) partially rescues sound-evoked potentials. These data suggest that the interaction of PI(4,5)P_2 with dTULP is crucial for the appropriate localization and function of dTULP-regulated ciliary membrane proteins.

DISCUSSION

In this study, we present evidence that PI(4,5)P_2 in the ciliary membrane is an important regulator of ciliary membrane protein trafficking. Specifically, the PI(4,5)P_2 in the ciliary base, itself regulated by the 5-phosphatase dINPP5E, binds to and regulates the function of dTULP, another regulator of ciliary protein trafficking. Mutations in *dInpp5e* elevate PI(4,5)P_2 levels and lead to ciliary accumulation of dTULP and mislocalization of IAV and NOMPC (Figures 1G, 2A, and 2B). In addition, ectopic expression of Skittles, a kinase that phosphorylates PI4P to PI(4,5)P_2, also increases ciliary PI(4,5)P_2 and phenocopies the ciliary *dInpp5e* phenotypes (Figure 3C). These results confirm that the *dInpp5e* mutant phenotype is caused primarily by an increase in ciliary PI(4,5)P_2.

In *Drosophila* chordotonal neurons, dINPP5E is localized in the ciliary compartment known as the transition zone, which marks the boundary between the plasma membrane and the ciliary membrane. This highly specific localization is consistent with a role for dINPP5E in controlling the entry and exit of ciliary proteins via its regulation of PIP levels. We thus propose that dINPP5E acts as a gate keeper for membrane PIPs preventing diffusion of PI(4,5)P_2 into the ciliary membrane from the plasma membrane by continuously hydrolyzing PI(4,5)P_2 to PI4P.

Previously, we suggested dTULP may deliver to IFT particles in the ciliary base pre-ciliary vesicles containing specific types of membrane proteins (e.g., IAV); vesicles perhaps "tagged" by the presence of PI(4,5)P_2. In this study, we report that increasing PI(4,5)P_2 in the ciliary base, either by mutation of *dInpp5e* (Figure 3A) or by ectopic expression of the Skittles (Figure 3C), dramatically enhances the ciliary accumulation of both IAV and dTULP. Mutation of the dTULP PIP-binding domain, however, partially rescues the abnormal ciliary IAV accumulation caused by elevated PI(4,5)P_2 as well as the resulting hearing defects. Together, these data suggest that the level of interaction between dTULP and PI(4,5)P_2 in the ciliary base is a determining factor in IAV and dTULP trafficking. One possibility is that the PIP- and IFT-binding domains of dTULP work cooperatively such that the binding of dTULP to PI(4,5)P_2 alters the affinity of the IFT-binding domain for the IFT machinery. This could explain the enhanced ciliary accumulation of dTULP under conditions of elevated PI(4,5)P_2.

IAV and NOMPC are TRP channels that are present in chordotonal organs and are essential for hearing. Each channel has a characteristic localization in the cilia of chordotonal neurons, and the preservation of this distribution is important for normal sound transduction. However, little is known about the *cis*-acting and *trans*-acting factors that affect their ciliary trafficking. dTULP is one *trans*-acting factor required for ciliary delivery of IAV (Park et al., 2013). NOMPC, in contrast, is only redistributed from distal to proximal cilia in the absence of dTULP (Park et al., 2013). Thus, we expect that other *trans*-acting factors are required for its ciliary localization. Further studies of the ciliary localization sequences in channels themselves and of the *trans*-acting factors that detect them will clarify our understanding of TRP channel ciliary trafficking.

Just before we submitted this study, two other groups reported on the role mammalian INPP5E plays in regulating ciliary membrane composition (Chávez et al., 2015; Garcia-Gonzalo et al., 2015). Mutations in *Inpp5e* increase ciliary accumulation of TULP3 and GPR161, and ciliary GRP161 antagonizes hedgehog signaling during development. These parallels in a mammalian system with our results in *Drosophila* are clear evidence that the role of ciliary INPP5E we report here is well conserved across species. There are, however, a few key points of difference between these studies and our own. First, PI4P is the main PIP found in the primary cilia of mammalian cells, and INPP5E inactivation leads to an extension of the localization of PI(4,5)P_2 all the way to the ciliary tip. In *Drosophila* chordotonal cilia, PI4P levels are very low (data not shown), and the loss of dINPP5E increases the level of PI(4,5)P_2 only in a well-defined patch near the ciliary base. This is presumably due to the tight localization of dINPP5E near the ciliary base. We expect that this new spatial information from *Drosophila* will help sort out any distinctions in ciliary subdomains where PIPs function in ciliary protein trafficking. Second, in mammals, TULP3 interacts with the IFT-A core complex (i.e., WDR19, IFT122, and IFT140) (Mukhopadhyay et al., 2010), and the ciliary TULP3 accumulation that occurs upon INPP5E inactivation also leads to ciliary IFT122 accumulation. We previously reported, however, that ciliary trafficking of dTULP is independent of IFT-A. In fact, even the extreme accumulation of dTULP in the dInpp5e mutants fails to cause ciliary RempA/IFT140 accumulation (Figure 2C). These results suggest that although some IFT components are essential for the dTULP-regulated ciliary trafficking of various membrane receptors, dTULP's interaction with PIPs is more important than its interaction with the IFT machinery for its own ciliary localization, at least in the chordotonal neurons. Third, here we demonstrate that changes in the PIP concentration can lead to physiological changes at the level of organ function—in this case, the chordotonal organ that allows *Drosophila* to hear. As far as we know, ours is the first report linking distinct changes in ciliary PIPs to phenotypic changes in vivo.

The present study raises several interesting questions for further study. Although INPP5E clearly plays a role in ciliary regulation, it is unclear whether it and other PIP-modifying enzymes are being actively regulated in cilia themselves. Any relevant conditions that modulate the level or activity of INPP5E could alter the sensitivity of cilia to external stimuli or even the context of signaling pathways activated by cilia-specific receptors (Plotnikova et al., 2015). Since our study uncovered differences in the relationships of various cilia-specific proteins to the PIP-modulated trafficking protein dTULP, it will be interesting to see whether molecules other than dTULP/TULP3 that also have PIP-binding domains regulate complementary subsets of cilia-specific proteins.

If so, the role of various PIPs in the regulation of ciliary functions under normal and disease conditions will expand.

EXPERIMENTAL PROCEDURES

Fly Stocks

dTulp^{1}, *UAS-dTulp*^{wt}, and *UAS-dTulp*$^{PIP-}$ were previously described (Park et al., 2013). *Iav-GFP*, *RempA-YFP,* and *NompB-GFP* were gifts from M. Kernan (Han et al., 2003; Kim et al., 2003; Lee et al., 2008). We obtained *dInpp5e*^{MI} (MI02221;BL33177), *UAS-Sktl*, *F-GAL4*, *elav-GAL4*, *UAS-mCD8:GFP*, *nos-Cas9,* and *UAS-Dcr2* from the Bloomington Stock Center. We obtained RNAi lines specific to *skittles* (BL27715), *Pi3K92E* (BL27690), *Ocrl* (BL34722), *dInpp5e* (BL34037, v16048), and *Pten* (BL25841) from the Vienna *Drosophila* RNAi Center and the Bloomington Stock Center. w^{1118} was used as a control. All knockout alleles and transgenic lines except the RNAi lines were backcrossed for five generations to the w^{1118} control genotype.

Generation of *dInpp5e* Mutants and Transgenic Flies

We used the CRISPR/Cas9 system to generate the *dInpp5e*^{1} mutant allele (Gratz et al., 2013). Briefly, we selected two gRNAs specific to *dInpp5e* using TargetFinder (http://tools.flycrispr.molbio.wisc.edu/targetFinder): GGATGTGG CTCCACCTTACTAGG (+109 to +131) and GGATGACTAGTCCCTGCATTTGG (+2,034 to +2,056), where protospacer-adjacent motifs are underlined. These two *dInpp5e*-specific sequences were synthesized and cloned into the BbsI sites of PU6-BbsI-chiRNA to drive expression of a chimeric RNA (chiRNA) under the control of the *Drosophila* small nuclear RNA:U6:96Ab promoter. Two pU6-chiRNA targeting constructs were injected into *nos-Cas9* embryos at 250 ng/μl each to make larger defined deletions rather than small indels. Deletion lines were screened using PCR on genomic DNA isolated from the G_0 generation.

To make a genomic rescue line for use with the *dInpp5e* mutants, we obtained BAC clone CH322-17A13 from the BACPAC Resource Center. We then generated transgenic flies using PhiC31 integrase-mediated transgenesis on the II chromosome (Bloomington stock number 9723).

To make the EGFP-dINPP5E line, we subcloned from BAC clone CH322-17A13 a 7.1 kbp XbaI/SacII genomic DNA fragment encompassing the *dInpp5e* coding region (−3,178 to +3,969) into pBluescriptKSII+. This EGFP coding sequence was inserted in-frame into the first coding exon of *dInpp5e*. This EGFP tagged *dINPP5E* genomic fragment was inserted into the XbaI and SacII sites of the pCasper4 vector. The resulting construct was injected into *Drosophila* embryos, and resulting transgenics were selected according to standard techniques.

Electrophysiology

Sound-evoked potentials were recorded as described by Eberl et al. (Eberl et al., 2000). Briefly, the fly was immobilized in a trimmed pipette tip. The fly's antennal sound receivers were stimulated by computer-generated pulse songs delivered to the fly through Tygon tubing. Neuronal responses were detected using a recording electrode inserted into the junction between the first and second antennal segments, and a reference electrode was inserted into the dorsal head cuticle. The resulting signals were amplified with a DAM50 differential amplifier (World Precision Instruments) and digitized using a virtual instrument designed in LabVIEW (National Instruments). Each trace represents an average response to ten stimuli.

Immunohistochemistry

For whole-mount staining, antennae were dissected from late stage pupae (i.e., 36-48 hr after puparium formation). Dissected antennae were fixed for 15 min with 4% paraformaldehyde in 1X PBS containing 0.2% Triton X-100 (PBS-T) and washed three times with PBS-T. The fixed samples were blocked for 30 min with 5% heat-inactivated goat serum in PBS-T and incubated overnight at 4°C in primary antibodies diluted in the same blocking solution. The tissues were then washed three times for 10 min each with PBS-T and incubated for 1 hr at room temperature in secondary antibodies diluted 1:500 in blocking solutions. Following three washes in PBS-T, the samples were then mounted in Vectashield (Vector Laboratories) and examined using a Zeiss LSM700 confocal microscope (Jena).

When comparing localization and expression levels of ciliary proteins between control and experimental antennae, all samples were prepared at the same time and all confocal images were obtained under the same conditions. For quantification of ciliary protein levels, the pixel intensity corresponding to each protein was measured using Zen Software (Jena) and the mean pixel intensities from multiple scolopidia of several different samples are shown.

The following primary antibodies were used for immunohistochemistry at the following dilutions: rabbit anti-dTULP, 1:400; 22C10, 1:200 (Hybridoma Bank, University of Iowa); 21A6, 1:200 (Hybridoma Bank); rabbit anti-NOMPC, 1:400; rabbit anti-GFP, 1:1000 (Molecular Probes, Eugene, OR); mouse anti-GFP, 1:500 (Molecular Probes); mouse anti-PI(4,5)P_2, 1:100 (Abcam). The secondary antibodies were Alexa 488-, Alexa Fluor 568-, and Alexa Fluor 633-conjugated anti-mouse or anti-rabbit immunoglobulin G (Molecular Probes; 1:500). Actin was visualized with Alexa Fluor 633 Phalloidin (Molecular Probes).

Western Blot

Fly antennal lysates from each genotype were subjected to western blot analysis as previously described (Park et al., 2013). Rabbit anti-dTULP, 1:1000: anti-α-tubulin (Hybridoma Bank; 1:2,000).

Statistical Analyses

Plots in Figures 2F and 4E show the median ± interquartile range. Kruskal-Wallis tests with Mann-Whitney *U* post hoc tests was calculated in GraphPad Prism 5. Asterisks indicate statistical significance (**$p < 0.01$).

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.12.009.

AUTHOR CONTRIBUTIONS

Most of the experiments were performed by J.P. N.L. performed the electrophysiological analysis. A.K. analyzed the data and wrote the paper. J.T.S., C.H.K., and S.J.M. designed the experiments and wrote the paper.

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