Phosphoinositides Regulate Ciliary Protein Trafficking to Modulate Hedgehog Signaling

Graphical Abstract

Highlights

- The ciliary membrane contains different phosphoinositides than that of its base
- The ciliopathy-associated protein Inpp5e generates the phosphoinositide distribution
- Ciliary phosphoinositides are required for normal Hedgehog (Hh) signaling
- Tulp3 senses phosphoinositides to limit ciliary Gpr161, an inhibitor of Hh signaling

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In Brief

Garcia-Gonzalo et al. show that different domains of the ciliary membrane contain different phosphoinositides. The ciliopathy-associated enzyme Inpp5e controls this distribution, which is needed for trafficking of ciliary proteins, including Hedgehog signaling regulators Tulp3 and Gpr161. Disrupting phosphoinositide distribution impacts Hedgehog signaling, suggesting lipids are critical components of the cilia signaling environment.
Phosphoinositides Regulate Ciliary Protein Trafficking to Modulate Hedgehog Signaling

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http://dx.doi.org/10.1016/j.devcel.2015.08.001

SUMMARY

Primary cilia interpret vertebrate Hedgehog (Hh) signals. Why cilia are essential for signaling is unclear. One possibility is that some forms of signaling require a distinct membrane lipid composition, found at cilia. We found that the ciliary membrane contains a particular phosphoinositide, PI(4)P, whereas a different phosphoinositide, PI(4,5)P2, is restricted to the membrane of the ciliary base. This distribution is created by Inpp5e, a ciliary phosphoinositide 5-phosphatase. Without Inpp5e, ciliary PI(4,5)P2 levels are elevated and Hh signaling is disrupted. Inpp5e limits the ciliary levels of inhibitors of Hh signaling, including Gpr161 and the PI(4,5)P2-binding protein Tulp3. Increasing ciliary PI(4,5)P2 levels or conferring the ability to bind PI(4)P on Tulp3 increases the ciliary localization of Tulp3. Lowering Tulp3 in cells lacking Inpp5e reduces ciliary Gpr161 levels and restores Hh signaling. Therefore, Inpp5e regulates ciliary membrane phosphoinositide composition, and Tulp3 reads out ciliary phosphoinositides to control ciliary protein localization, enabling Hh signaling.

INTRODUCTION

Primary cilia are sensory organelles whose malfunction causes human diseases, known as ciliopathies (Hildebrandt et al., 2009; Qin et al., 2011; Tran et al., 2008; Liem et al., 2013; Norman et al., 2009; Patterson et al., 2009; Cameron et al., 2009; Qin et al., 2011; Tran et al., 2008; Liem et al., 2012). Although it is clear that the protein composition of the ciliary membrane is distinct from that of the surrounding, contiguous plasma membrane, it has been less clear how the lipid composition of the ciliary membrane differs from that of other cellular membranes. In Paramecia, the ciliary membrane is enriched in sphingolipids, and a mutation that alters ciliary lipid composition affects ciliary channel activity, suggesting that the ciliary lipid composition is critical for its function (Kaneshiro et al., 1984; Andrews and Nelson, 1979; Forte et al., 1981). Similarly, in Tetrahymena and Chlamydomonas, certain lipids are enriched in their cilia or flagella (Kennedy and Thompson, 1970; Jonah and Erwin, 1971; Smith et al., 1970; Gealt et al., 1981; Bloodgood et al., 1985). In Trypanosomes, the flagellum possesses high levels of sterols and saturated fatty acids and shows a high degree of lipid organization (Tyler et al., 2009; Souto-Padrón and de Souza, 1983). A region of high lipid organization also exists at the base of vertebrate epithelial cilia, indicating that subdomains within the cilium may differ in their lipid composition (Vieira et al., 2006; Montesano, 1979).

Different forms of another class of lipids, the phosphoinositides, help to define different cellular membranes (Di Paolo and De Camilli, 2006; Roth, 2004; Sasaki et al., 2009). In C. elegans, a phosphoinositide 5-phosphatase, CIL-1, controls PI(3)P levels and the ciliary localization of PKD-2, suggesting that phosphoinositides can participate in ciliary protein trafficking (Bae et al., 2009). In mammals, three phosphoinositide 5-phosphatases—Ocr1, Inpp5b, and Inpp5e—can localize to cilia (Jacoby et al., 2009; Bielas et al., 2009; Luo et al., 2012, 2013). Mutations in human INPP5E can cause the ciliopathy Joubert syndrome, and knockout of mouse Inpp5e results in phenotypes characteristic of ciliopathies, including cystic kidneys and polydactyly (Jacoby et al., 2009; Bielas et al., 2009). Therefore, we investigated whether distinct phosphoinositides were present in the ciliary membrane and whether they participate in the unique signaling functions of vertebrate cilia.
**RESULTS**

**PI(4)P and PI(4,5)P₂ Localize to Distinct Ciliary Compartments**

To investigate whether specific phosphoinositides localize to cilia, we expressed specific phosphoinositide-binding domains fused to fluorescent proteins in ciliated cells (Hammond and Balá, 2015). Unlike sensors for PI(3)P, PI(5)P, PI(3,4)P₂, PI(3,5)P₂, and PIP₂, a PI(4)P-specific sensor, EGFP-2xP4M₅ (Hammond et al., 2014), was enriched in cilia (Figure 1A and data not shown). EGFP-2xP4M₅ was present in 74% ± 4% of IMCD3 cilia. Line scans of ten such cilia reflect the presence of PI(4)P throughout the ciliary membrane (Figure 1B). Antibody staining confirmed the enrichment of PI(4)P within IMCD3 cilia (Figure S1A). Immunofluorescence also revealed that sea urchin cilia have abundant PI(4)P, indicating that PI(4)P is a component of the ciliary membrane in evolutionarily distant animals (Figure S1B).

Whereas PI(4)P was present along the length of cilia, a PI(4,5)P₂ sensor (EYFP-PH PLC<sup>ΔC₁</sup>) (Stauffer et al., 1998) localized to the proximal end of NIH 3T3 and IMCD3 cilia (Figure 1C; Figures S1C and S1D). EYFP-PH PLC<sup>ΔC₁</sup> fluorescence ceased at a sharp boundary near the ciliary base (Figure 1D). To confirm that EYFP-PH PLC<sup>ΔC₁</sup> fluorescence reflected PI(4,5)P₂ distribution, we targeted Inp54p, a yeast enzyme that specifically converts PI(4,5)P₂ to PI(4)P, to cilia by fusing it to a ciliary GPCR (Serotonin Receptor 6, 5HT₆) (Lin et al., 2013; Johnson et al., 2008; Tsujishita et al., 2001; Suh et al., 2006). Coexpression of 5HT₆-EYFP-Inp54p with a PI(4,5)P₂ sensor (mCerulean3-PI(4,5)P₂) reduced mCerulean fluorescence at the ciliary base (Figures S1E and S1F). A catalytically inactive version, 5HT₆-EYFP-Inp54p(D281A), did not affect mCerulean3-PI(4,5)P₂ localization (Figures S1E and S1F) (Suh et al., 2006). Conversely, targeting PI(4)P 5-kinase, type Iγ (PIPK) to cilia by fusing it to 5HT₆ expanded mCerulean3-PI(4,5)P₂ localization to the length of the cillum (Figures S1E and S1F) (Leno et al., 2011). Together, these data indicate that the ciliary membrane contains PI(4)P along its length, and PI(4,5)P₂ proximally.

**Inpp5e Generates Ciliary PI(4)P and Restricts Ciliary PI(4,5)P₂**

The transition zone, a region of the ciliary base, participates in protein localization to cilia (Czarnecki and Shah, 2012; Garcia-Gonzalo and Reiter, 2012). We found that Tctn1, a transition zone protein essential for vertebrate Hh signaling (Garcia-Gonzalo et al., 2011; Roberson et al., 2015), is required for the ciliary localization of Inpp5e (Figures 2A and 2B). Because Inpp5e can convert PI(4,5)P₂ into PI(4)P, we hypothesized that Inpp5e affects the relative levels of these lipids in the ciliary membrane. To test this hypothesis, we derived mouse embryonic fibroblasts (MEFs) from Inpp5e<sup>−/−</sup> and Inpp5e<sup>+/−</sup> embryos (Jacoby et al., 2009). As expected, Inpp5e was present in the cilia of Inpp5e<sup>−/−</sup> but not Inpp5e<sup>+/−</sup> MEFs (Figure S2A). Consistent with published data (Jacoby et al., 2009), Inpp5e<sup>−/−</sup> and Inpp5e<sup>+/−</sup> MEFs ciliated to equal extents (Figures S2B and S2C). To assess whether Inpp5e affects PI(4)P distribution, we examined the localization of the PI(4)P probe, EGFP-2xP4M<sup>ΔC₅</sup>, in Inpp5e<sup>−/−</sup> and Inpp5e<sup>+/−</sup> MEFs. EGFP-2xP4M<sup>ΔC₅</sup> localization to cilia was severely reduced in Inpp5e<sup>−/−</sup> MEFs, further suggesting that EGFP-2xP4M<sup>ΔC₅</sup> distribution accurately reflects PI(4)P distribution and indicating that Inpp5e is important for generating ciliary PI(4)P (Figures 2C and 2D).

As Inpp5e has the ability to remove the 5-phosphate from PI(4,5)P₂, we hypothesized that it regulates ciliary PI(4,5)P₂ levels. To test this, we expressed mCerulean3-PI(4,5)P₂<sup>ΔC₁</sup>, a PI(4,5)P₂ probe, in Inpp5e<sup>−/−</sup> and Inpp5e<sup>+/−</sup> MEFs. Similarly to NIH 3T3 and IMCD3 cells, Inpp5e<sup>−/−</sup> MEFs localized mCerulean3-PI(4,5)P₂<sup>ΔC₁</sup> at the proximal cillum (Figures 2E and 2F). In
In contrast, Inpp5e<sup>−/−</sup> MEFs localized mCerulean3-PH<sup>PLC<sub>G1</sub></sup> along the entire length of the ciliary membrane (Figures 2E and 2F). Thus, Inpp5e limits ciliary PI(4,5)P<sub>2</sub> and generates ciliary PI(4)P within cilia.

To test whether the expanded ciliary PI(4,5)P<sub>2</sub> in Inpp5e<sup>−/−</sup> MEFs reflects loss of ciliary PI(4,5)P<sub>2</sub> 5-phosphatase activity, we examined whether targeting the yeast PI(4,5)P<sub>2</sub> 5-phosphatase Inp54p to cilia lacking Inpp5e restored normal ciliary PI(4,5)P<sub>2</sub> levels. 5HT<sub>6</sub>-EYFP-Inp54p, but not the catalytically inactive 5HT<sub>6</sub>-EYFP-Inp54p(D281A), restored the ciliary exclusion of mCerulean3-PH<sup>PLC<sub>G1</sub></sup> in Inpp5e<sup>−/−</sup> MEFs (Figure S2D). These results further suggest that Inpp5e dephosphorylates ciliary PI(4,5)P<sub>2</sub> to restrict it to the proximal cilium.

Because Tctn1 is required to localize Inpp5e to cilia, we hypothesized that, like Inpp5e<sup>−/−</sup> MEFs, Tctn1<sup>−/−</sup> MEFs would demonstrate altered ciliary phosphoinositide composition. As with other cell types, the PI(4,5)P<sub>2</sub> probe mCerulean3-PH<sup>PLC<sub>G1</sub></sup> was restricted to the base of Tctn1<sup>+/+</sup> MEF cilia, in contrast to the wild-type MEFs, but like Inpp5e<sup>−/−</sup> MEFs, mCerulean3-PH<sup>PLC<sub>G1</sub></sup> localized along the full length of Tctn1<sup>−/−</sup> MEF cilia (Figures S2E and S2F). This indicates that, like Inpp5e, Tctn1 is required for the localization of PHPLC to cilia. These results suggest that Tctn1 is not required for the localization of other PHPLC probes, such as PH-PLC<sub>B1</sub> or PH-PLC<sub>B2</sub>, to cilia. Therefore, we examined whether targeting the yeast PI(4,5)P<sub>2</sub> 5-phosphatase Inp54p to cilia lacking Tctn1 restored normal ciliary PI(4,5)P<sub>2</sub> levels. 5HT<sub>6</sub>-EYFP-Inp54p, but not the catalytically inactive 5HT<sub>6</sub>-EYFP-Inp54p(D281A), restored the ciliary exclusion of mCerulean3-PH<sup>PLC<sub>G1</sub></sup> in Tctn1<sup>−/−</sup> MEFs (Figure S2D). These results further suggest that Inpp5e dephosphorylates ciliary PI(4,5)P<sub>2</sub> to restrict it to the proximal cilium.

Inpp5e<sup>−/−</sup> MEFs with the N-terminal signaling portion of Shh, ShhN, or a pharmacological agonist of Smo, SAG, robustly increased transcription of Gli1 and Ptc1, two Hh target genes (Figure 3A; Figures S3A–S3C) (Chen et al., 2002). These responses were abrogated in mutant Inpp5e<sup>−/−</sup> MEFs, revealing a role for Inpp5e in promoting Hh signaling (Figure 3A and Figures S3A–S3C).

To investigate how the Inpp5e-mediated control of ciliary phosphoinositide levels participates in Hh signaling, we examined the subcellular localization of Hh pathway components in control and Inpp5e<sup>−/−</sup> MEFs. Because Inpp5e is required for cells to respond to the Smo agonist SAG (Figure 3A), ciliary phosphoinositides are likely to participate in Hh signaling at the level of, or downstream of, Smo. As expected, Ptc1 localized normally to cilia in Inpp5e<sup>−/−</sup> MEF (Figure S3D) (Rohatgi et al., 2007). Because CIL-1, a C. elegans homolog of Inpp5e, helps control the ciliary localization of PKD-2, another ciliary membrane protein and the ortholog of mammalian Polycystin-2 (Bae et al., 2009), we examined whether Inpp5e also affects the ciliary localization of Polycystin-2. Unlike nematodes, mouse Inpp5e is dispensable for the ciliary localization of Polycystin-2.
Similarly, Inpp5e was not required either for the exclusion of Smo from cilia in the absence of pathway activation or for its ciliary localization upon stimulation with SAG (Figures 3B and 3C and Figure S3F). In contrast, the SAG-dependent accumulation of Gli3 at the ciliary tip was reduced in Inpp5e−/− cells (Figures 3D and 3E and Figure S3G). Therefore, Inpp5e promotes Hh signal transduction at a step subsequent to Smo ciliary localization and prior to Gli3 accumulation at the ciliary tip.

Recently, Gpr161 was identified as a negative regulator of Hh signaling that functions upstream of Gli transcription factor accumulation in cilia (Mukhopadhyay et al., 2013). Remarkably, we observed that ciliary levels of Gpr161 in Inpp5e−/− MEFS were greater than in Inpp5e+/− MEFS (Figures 3F and 3G). SAG treatment lowered ciliary Gpr161 levels approximately 3-fold in both cell types relative to unstimulated cells, as a result of which Gpr161 levels remained higher in Inpp5e−/− MEFS after SAG treatment (Figure 3G and Figure S3H). Thus, Inpp5e limits ciliary Gpr161 levels but does not prevent the Hh-dependent ciliary exit of Gpr161.

**Inpp5e Limits the Ciliary Localization of Tulp3 and IFT-A**

Because the ciliary localization of Gpr161 requires Tulp3, a protein that binds PI(4,5)P2 and that, like Gpr161, restrains vertebrate Hh signaling (Mukhopadhyay et al., 2010, 2013), we examined whether Inpp5e affects Tulp3 localization. Consistent with a previous report (Norman et al., 2009), Tulp3 was only occasionally observed within control cilia (Figures 4A and 4B). In contrast, Tulp3 robustly accumulated along the full length of...
Inpp5e−/− MEF cilia (Figures 4A and 4B). Tulp3 was also present at abnormally high levels in the primary cilia of Inpp5e−/− E9.5 neural tubes and other tissues (Figures 4C and 4D and data not shown). Immunoblot analysis revealed that Tulp3 levels are equivalent in Inpp5e+/− and Inpp5e−/− MEFs (Figure S4 A), suggesting that ciliary phosphoinositides affect Tulp3 localization to cilia but not its stability.

Tulp3 interacts with the IFT-A complex, and IFT-A components such as Ift139 (also called Thm1 or Ttc21b) restrain vertebrate Hh signaling, similar to Tulp3 and Gpr161 (Qin et al., 2011; Tran et al., 2008; Liem et al., 2012). Therefore, we investigated whether Inpp5e also restricts the ciliary localization of IFT-A components. Like Tulp3, IFT-A components Ift139 and Ift140 overaccumulated in the cilia of Inpp5e−/− MEFs (Figures 4E and 4F and Figure S4B). In contrast, loss of Inpp5e did not affect the ciliary levels of IFT-B component Ift140 (Figure S4C). Thus, in addition to promoting Hh signaling, Inpp5e limits the ciliary localization of the Hh-signaling negative regulators Ift139, Ift140, Tulp3, and Gpr161. Because these proteins interact with each other, we hypothesized that the phosphoinositide levels controlled by Inpp5e limit their localization to cilia.

Since Tctn1−/− MEF cilia, like those of Inpp5e−/− MEFs, lack Inpp5e and accumulate PI(4,5)P2, we tested whether they also accumulate Tulp3 and Gpr161. Ciliary levels of Tulp3 in Tctn1−/− MEFs were not increased (Figure S4D), and ciliary levels of Gpr161 were decreased in Tctn1−/− MEFs (Figure S4E). Given that many other membrane-associated proteins, such as Polycystin-2 and Smo, fail to localize to cilia in Tctn1−/− MEFs but localize normally to the cilia of Inpp5e−/− MEFs (Garcia-Gonzalo et al., 2011 and this work), we conclude that the disruption of the ciliary gate at the transition zone caused by loss of Tctn1 leads to a more pervasive defect in localizing ciliary membrane-associated proteins than does absence of ciliary Inpp5e.

Inpp5e Limits Ciliary Tulp3 and IFT-A Levels by Restricting Ciliary PI(4,5)P2

To determine whether the ciliary buildup of Tulp3 in Inpp5e−/− cells was due to the elevated ciliary PI(4,5)P2 levels, we tested whether expression of wild-type or catalytically dead Inpp5e affected ciliary Tulp3 levels. Expression of EGFP-INPP5E-WT in Inpp5e−/− cilia removed Tulp3 from cilia, whereas catalytically inactive EGFP-INPP5E-D477N had no effect, consistent with a phosphatase-dependent role for Inpp5e in controlling ciliary Tulp3 levels (Figures 5A and 5B).

To assess whether PI(4,5)P2 is sufficient to cause Tulp3 to accumulate in cilia, we targeted a PI(4)P 5-kinase (5HT 6-EYFP-PIPK) to the cilia of wild-type IMCD3 cells. As shown previously, 5HT6-EYFP-PIPK increases ciliary PI(4,5)P2 levels (Figures S1E and S1F).
and S1F). In addition to increasing ciliary PI(4,5)P$_2$ levels, 5HT$_6$-EYFP-PIPK increased ciliary Tulp3 levels (Figures 5C and 5D). In contrast, the inactive mutant 5HT$_6$-EYFP-PIPK-D253A or 5HT$_6$-EYFP-Inp54p had no such effect (Figures 5C and 5D).

Because Tulp3 is required for the ciliary delivery of Gpr161, we tested whether increasing ciliary PI(4,5)P$_2$ levels also increased ciliary Gpr161 localization. Expression of 5HT$_6$-EYFP-PIPK caused a modest but significant increase in ciliary Gpr161 levels as compared to expression of 5HT$_6$-EYFP-PIPK-D253A (Figures S5A and S5B). Because increasing ciliary PI(4,5)P$_2$ levels—either by removing Inpp5e or by increasing ciliary PI(4)P 5-kinase activity—increased ciliary levels of Tulp3 and Gpr161, we conclude that maintaining low ciliary PI(4,5)P$_2$ levels is critical for restraining ciliary Tulp3 and Gpr161 localization.

Tulp3 binds PI(4,5)P$_2$ but not PI(4)P (Mukhopadhyay et al., 2010). We hypothesized that this phosphoinositide binding specificity accounted for the limited localization of Tulp3 to cilia of wild-type cells and the increased localization of Tulp3 to cilia with increased PI(4,5)P$_2$ levels. To test this hypothesis, we generated a Tulp3 with altered phosphoinositide binding specificity by fusing Tulp3-EGFP to P4MSidM, a PI(4)P-binding domain of a Legionella protein (Hammond et al., 2014). Whereas, like endogenous Tulp3, Tulp3-EGFP weakly localized to cilia, Tulp3-EGFP-P4MSidM, like Tulp3 in cilia with elevated PI(4,5)P$_2$ levels, robustly localized to cilia (Figures 5E and 5F). These results suggest that persistent interaction with phosphoinositides of the ciliary membrane is sufficient to increase the ciliary localization of Tulp3 and that increased ciliary PI(4,5)P$_2$ levels are sufficient to increase ciliary localization of Tulp3.

To assess whether altered ciliary Tulp3-phosphoinositide interactions are sufficient to affect the localization of Tulp3-interacting proteins, we examined whether Tulp3-EGFP-P4MSidM affected the ciliary localization of Ift140. Expression of Tulp3-EGFP-P4MSidM caused Ift140 to accumulate in cilia (Figures 5E and 5F), similar to the accumulation of Ift140 in Inpp5e$^{-/-}$ cells, whereas expression of Tulp3-EGFP had no effect on ciliary Ift140. These results are consistent with differential Tulp3 interaction with PI(4,5)P$_2$ in the membrane of the ciliary base and PI(4)P in the more distal ciliary membrane being critical to limit the ciliary localization of both Tulp3 and its interacting protein, Ift140.

**Inhibition of Tulp3 or Gpr161 Increases Hh Signaling in Inpp5e$^{-/-}$ MEFs**

We hypothesized that the Hh signaling defects caused by loss of Inpp5e were due to the PI(4,5)P$_2$-dependent increase in ciliary Tulp3 and its associated proteins, including Gpr161. We reduced Tulp3 and Gpr161 levels in Inpp5e$^{-/-}$ MEFs using small interfering RNA (siRNA)-mediated knockdown (Figures S6A–S6C). Reducing Tulp3 reduced the ciliary levels of Gpr161, consistent with a role for Tulp3 in delivering Gpr161 to the cilium (Figures S6A and S6C). In contrast, reducing Gpr161 had no...
limiting ciliary PI(4,5)P₂ levels is critical for restricting the localization of the small GTPase Arl13b to the cilium and other components of the transition zone MKS complex to confine the Inpp5e phosphatase to the cilium (Humbert et al., 2012). Thus, it is likely that Tctn1 and the transition zone MKS complex localize Inpp5e to cilia through their effects on Arl13b.

In the absence of Inpp5e, ciliary levels of PI(4)P decrease and levels of PI(4,5)P₂ increase, suggesting that Inpp5e is critical for generating the observed distribution of ciliary phosphoinositides. It will be interesting to determine whether Inpp5e shares this function with other ciliary phosphoinositide phosphatases, such as Ocrl or Inpp5b (Luo et al., 2012, 2013).

Perturbing the ciliary levels of ciliary PI(4)P and PI(4,5)P₂, either by removing Inpp5e or by targeting the phosphoinositide 5-kinase Pipk to cilia, is sufficient to increase the ciliary localization of Tulp3. Because Tulp3 interacts with PI(4,5)P₂ but not PI(4)P (Mukhopadhyay et al., 2010), it is an excellent candidate for distinguishing between these two domains within the cilium. Tulp3 binds to the IFT-A complex to deliver GPCRs such as Gpr161 to cilia (Mukhopadhyay et al., 2010, 2013). Consistent with a critical role for Tulp3-mediated sensing of phosphoinositides in this process, disruption of the ciliary phosphoinositide distribution not only increases ciliary Tulp3 but also increases the ciliary levels of IFT-A components and Gpr161. Although Tulp3 is one key interpreter of ciliary phosphoinositides, it may not be the sole one. Other Tubby family proteins may share this role. Mutation of either Tubby or Tulp1 causes retinal degeneration, a phenotype commonly observed in ciliopathies (Mukhopadhyay and Jackson, 2011). By analogy to our findings with Tulp3 and Gpr161, it is possible that photoreceptor phosphoinositides are read out by Tubby and Tulp1 to control the delivery of Rhodopsin to the outer segment, defects that cause photoreceptor loss.

Tulp3, IFT-A components such as Ift139, and Gpr161 are all negative regulators of ciliary Hh signaling (Mukhopadhyay et al., 2013; Norman et al., 2009; Patterson et al., 2009; Cameron et al., 2009; Qin et al., 2011; Tran et al., 2008; Liem et al., 2012). In addition to limiting ciliary PI(4,5)P₂ levels, Inpp5e limits the ciliary localization of these negative regulators and is required for normal Hh signal transduction. Reducing Tulp3 levels in cilia using an siRNA restores Hh signaling in MEFs lacking Inpp5e, suggesting that overaccumulation of Tulp3 in cilia is a key factor in the observed signaling defects. The effect of the Tulp3 siRNA on Hh signaling is consistent with the effect of loss-of-function mutations in Tulp3 on developmental Hh signaling, suggesting that the siRNA effect is on target, but it will be of interest to confirm this epistasis using genetic tools (Norman et al., 2009; Patterson et al., 2009; Cameron et al., 2009). Reducing Gpr161 levels also increased Hh signaling in Inpp5e⁻/⁻ MEFs, suggesting that it is also sufficient to limit normal Hh signaling. The more modest effect of reducing Gpr161 levels, reflected by the fact that the fold induction of Ptch1 by SAG was unaffected by Gpr161 depletion, raises the possibility that overaccumulation of Tulp3 in cilia also causes the ciliary accumulation of additional negative regulators of Hh signaling. Our finding that reducing Tulp3 or Gpr161 levels increases Hh response even in the presence of increased ciliary PI(4,5)P₂ indicates that control of these two protein levels is a critical role for ciliary phosphoinositides in Hh signaling.

In mammalian cells, Tulp3 interprets ciliary phosphoinositides to control the localization of interactors such as IFT-A components and Gpr161. Increasing the ciliary levels of PI(4,5)P₂ (by loss of Inpp5e or by expressing a ciliary phosphoinositide phosphatase) may limit the ability of the cilium to transduce Hh signals.

**DISCUSSION**

Phosphoinositides help confer identity to organelles. For example, endosome and Golgi membranes possess PI(3)P and PI(4)P, respectively (Di Paolo and De Camilli, 2006; Roth, 2004), whereas the plasma membrane possesses both PI(4)P and PI(4,5)P₂ (Hammond et al., 2012, 2014). Physical separation of these membrane-bound organelles makes their distinct lipid compositions possible. In contrast to most organelles, the cilium is not membrane-bounded. Despite this difference, we found a sharp boundary at the cilium base that separates the ciliary membrane into a distal PI(4)P-containing domain and a proximal PI(4,5)P₂-containing domain.

Creating this phosphoinositide boundary depends on the Tctn1 complex of the transition zone to confine the Inpp5e phosphatase within the cilium. We previously showed that Tctn1 and other components of the transition zone MKS complex are critical for localizing the small GTPase Arl13b to the cilium (Garcia-Gonzalo et al., 2011). Arl13b is itself critical for the ciliary localization of Inpp5e (Humbert et al., 2012). Thus, it is likely that Tctn1 and the transition zone MKS complex localize Inpp5e to cilia through their effects on Arl13b.

In addition to decreasing ciliary Gpr161 levels, depletion of Tulp3 increased SAG-activated Hh signaling in Inpp5e⁻/⁻ MEFs (Figure S6D). Depletion of Gpr161 in Inpp5e⁻/⁻ MEFs led to a more modest but still significant increase in SAG-activated Hh signaling (Figures S6D and S6E). We conclude, therefore, that limiting ciliary PI(4,5)P₂ levels is critical for restricting the ciliary localization of Tulp3 and other negative regulators of Hh signaling (Figure 6). Defects in maintaining the normal distribution of ciliary phosphoinositides alters ciliary trafficking of Tulp3, IFT-A, and Gpr161, disrupting ciliary Hh signaling.

**Figure 6. Model of the Role of Ciliary Phosphoinositides in Hh Signaling**

Inpp5e restricts PI(4,5)P₂ levels in the ciliary membrane. The ability of Tulp3 to interact with PI(4,5)P₂ but not PI(4)P is critical for limiting its accumulation and that of its interactors IFT-A and Gpr161 within the cilium. In the absence of Inpp5e, ciliary PI(4,5)P₂ levels increase, increasing the amount of negative regulators of Hh signaling. Tulp3, IFT-A, and Gpr161, within the cilium and restricting the ability of the cilium to transduce Hh signals. See also Figure S6.
5-kinase) or fusing Tulp3 with a PI(4)P-binding domain increases the ciliary localization of Tulp3 and IFT-A components. Therefore, restricting ciliary PI(4,5)P₂ levels is critical to limiting the ciliary localization of Tulp3 and its interactors. Thus, the interplay between proteins and lipids helps generate the specialized subcellular environment present in the primary ciliary and is critical for its ability to transduce signals.

In most Drosophila cells, the Hh pathway functions independently of cilia, yet PI(4)P is required for Drosophila Hh signaling (Yavari et al., 2010). Although the cellular contexts may be different, a membrane domain high in PI(4)P and low in PI(4,5)P₂ (Yavari et al., 2010). Although the cellular contexts may be different of cilia, yet PI(4)P is required for

EXPERIMENTAL PROCEDURES

Animal Models and Cell Lines
Inpp5e⁻/⁻ mice have been described previously, as have Tctn1⁻/⁻ mice and MEFs (Jacoby et al., 2009; García-Gonzalo et al., 2011; Reiter and Skarnes, 2008). We derived MEFs from littermate E19.5 Inpp5e⁺/⁺ and Inpp5e⁻/⁻ embryonic tails. Briefly, tails were dissected, rinsed in Dulbecco’s PBS containing penicillin and streptomycin (PenStrep), digested for 10 min in 0.05% Trypsin-EDTA, disaggregated by pipetting, and plated on DMEM medium supplemented with 20% FBS and PenStrep. These primary MEFs were maintained in DMEM, 15% FBS, and PenStrep and immortalized by infection with a lentivirus expressing SV40 large T antigen. All MEF experiments in this study were performed using immortalized MEFs grown in DMEM and 10% FBS. All mouse protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, San Francisco.

For cilia isolation from sea urchin embryos, eggs and sperm from adult Strongylocentrotus purpuratus (Kerckhoff Marine Lab) were collected, combined, and cultured for 2 days in natural seawater at ~10,000 embryos/ml at 12°C with constant stirring. Midgastrula embryos were collected, concentrated by centrifugation, and washed three times with natural seawater. Pelleted embryos were resuspended in natural seawater + 0.5M NaCl to amputate cilia. Deciliated embryos were removed from the sample by centrifugation at 400 × g for 5 min. Cilia were then pelleted from the supernatant by centrifugation at 10,000 × g, 20 min, 4°C.

Immunofluorescence
Antibodies used in this study were: goat anti-γ-Tubulin (Santa Cruz, sc-7396), chicken anti-EGFP (Aves Labs, GFP-1020), mouse anti-acetylated tubulin (Sigma, 6-11B-1), anti-Arl13b (NeuroMab, 75-287), anti-Gli3 (GlisN-6FS) (Wen et al., 2010), anti-Pi4P (Echelon Bioscience, Z-P004), rabbit anti-Inpp5e (Jacoby et al., 2009), anti-Arl13b (Casparry et al., 2007), anti-Smoothenin (Abcam, ab38686), anti-Patched1 (Rohati et al., 2007), anti-Tul3p (Norman et al., 2009), anti-Tfl40 (ProteinTech, 17460-1-AP), and anti-Gpr161 (ProteinTech, 13398-1-AP and Mukhopadhyay et al., 2013). For immunostaining of MEFs, cells were grown on coverslips and fixed with 4% PFA in PBS for 5–10 min at room temperature (RT) followed by 3 min at ~20°C in cold methanol. PFA fixation was omitted when staining for Ift140. Pi4P antibody was used according to the manufacturer’s instructions. For Tul3p staining, cells were fixed for 10 min at 4°C in acetone. After fixation, cells were blocked in PBS containing 0.1% Triton X-100 and 2% donkey serum. For chicken EGFP staining, cells were also blocked in BlokHen-II reagent (Aves Labs) diluted in PBS. After blocking, cells were incubated in block containing primary antibodies for 1 hr at 37°C, 3 hr at RT, or overnight at 4°C. Coverslips were then rinsed twice in PBS, incubated with secondary antibodies and Hoechst 33342 or DAPI (30 min at 37°C or 1 hr at RT), rinsed twice in milliQ water, and mounted in gelvatol. For mouse embryo analyses, we fixed dissected embryos for 1–2 hr in 4% PFA in PBS, washed three times in PBS, and sunk overnight in 30% sucrose. Embryos were then equilibrated and frozen in OCT compound (Sakura) using a dry ice-ethanol bath. For staining, cryosections were thawed and washed three times in PBST (PBS with 0.1% Triton), encircled with an imEdge hydrophobic pen, and blocked and stained with antibodies as above. All imaging was performed using a Leica TCS SPE confocal microscope except for Figure 1A, where a Nikon N-SIM Ti-E microscope was used.

Live-Cell Imaging
NIH 3T3, mIMCD3, and MEFs were cultured in DMEM (GIBCO) supplemented with 10% FBS. For all transient transfections, cells were transfected with the respective DNA constructs by plating them directly in a transfection solution with 10% FBS. For all transient transfections, cells were transfected with the respective DNA constructs by plating them directly in a transfection solution containing DNA plasmid and Xtremegrane 9 (Roche). Cells were plated on poly(D-lysine)-coated borosilicate glass Lab-Tek 8-well chambers (Thermo Scientific). Ciliaogenesis was induced by serum starvation for 24 hr. Live-cell imaging was mostly performed using an IX-71 (Olympus) microscope with a 40× oil objective (Olympus) (with additional 1.6× optical zoom) and a Cool- SNAP HQ charge-coupled device camera (Photometrics). Micrographs were taken using MetaMorph 7.5 imaging software (Molecular Devices).

DNA Constructs
To construct the 5HT₆-EYFP expression plasmid, we amplified DNA encoding 5HT₆ flanked by 5’ and 3’ AgeI cleavage sites by PCR from 5HT₆-EGFP (Barbieri et al., 2009) and subcloned it into pEYFP-C1 (Clontech). To construct the 5HT₆-EYFP-PiPK, 5HT₆-EYFP-PiPK(D253A), 5HT₆-EYFP-Inp54p, and 5HT₆-EYFP-Inp54p(D281A) expression plasmids, we digested DNAs encoding the wild-type or mutant forms of PiPK or Inp54p from CFP-FKBP-PiPK or CFP-FKBP-Inp54p (Suh et al., 2006) with 5’ EcoRI and 3’ BamH cleavage sites and subcloned them into the 5HT₆-EYFP expression plasmid. To generate pEFP-Tul3p-P4M5SidM, we amplified the Tul3p ORF flanked by 5’ BglII and 3’ XhoI sites by PCR from pGLAP3-Tul3p (Mukhopadhyay et al., 2013) and subcloned it into pEFP-2xP4M5SidM (Hammond et al., 2014). We created pEFP-Inp54p-D477N from pEFP-Inp54p-WT (Jacoby et al., 2009) using site-directed mutagenesis (QuikChange, Agilent).

Hh Signal Transduction Assay
MEFs were plated on 12-well plates in full medium and allowed to reach confluency. The medium was replaced by OptiMEM containing either vehicle, 200 nM SAG (Cayman Chemicals), or 1 μg/ml mouse recombinant Shh-N-C25II (R&D Institute).
along the length of each cilium (Sensorcilium), and a second identical line of cilia length. For phosphoinositide sensor signal line scans, a line was traced along the length of each cilium (Sensor_cilium), and a second identical line (Sensor_background) was traced in close proximity to the first line within the cell area. Normalized values of (Sensor_cilium – Sensor_background) were plotted against relative distance along the cilium and fitted to sigmoid curves if appropriate.

Quantitation of ciliary signal intensities was carried out using ImageJ software. Each experiment was performed at least three times. Z stacks of seven fields of cells were acquired from each condition using a 63× objective and 1.5× digital zoom in a Leica TCS SPE confocal microscope. For each field, a maximal z projection was created and cilia were identified using Arl13b or Inpp5e, and cilia length was measured analogously and expressed as percentage relative to cilia length. For phosphoinositide sensor signal line scans, a line was traced along the length of each cilium (Sensor_cilium), and a second identical line (Sensor_background) was traced in close proximity to the first line within the cell area. Normalized values of (Sensor_cilium – Sensor_background) were plotted against relative distance along the cilium and fitted to sigmoid curves if appropriate.

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SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2015.08.001.

AUTHOR CONTRIBUTIONS

F.R.G., S.C.P., T.J., and J.F.R. devised all the experiments, most of which were executed by F.R.G. and S.C.P. Western blots and SIM imaging were performed by E.C.R. and G.G., respectively. Sea urchin flagella and Inpp5e mutant mice were generated by M.A. and S.S., respectively. F.R.G., S.C.P., T.J., and J.F.R. wrote the manuscript. T.I. and J.F.R. supervised the work.

ACKNOWLEDGMENTS

This work was supported by grants from the NIH (AR054396 and GM095941) and DK102910 to J.F.R. and DK102910 to T.I. and from the Burroughs Wellcome Fund, the Packard Foundation, and the Sandler Family Supporting Foundation (to J.F.R.). S.C.P. is supported by the Agency for Science, Technology and Research in Singapore.

Received: July 9, 2015
Revised: August 1, 2015
Accepted: August 5, 2015
Published: August 24, 2015

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Note Added in Proof