INPP5E regulates phosphoinositide-dependent cilia transition zone function

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Abbreviations used: DSHB, Developmental Studies Hybridoma Bank; E, embryonic day; Hh, Hedgehog; JBTS, Joubert syndrome; MEF, mouse embryonic fibroblast; MFI, mean fluorescence intensity; PI, phosphoinositide; PI3K, phosphoinositide 3-kinase; pMEF, primary MEF; Ptch, Patched; ROI, region of interest; RPE, retinal pigment epithelial; SAG, smoothened agonist; Shh, Sonic Hedgehog; SMO, Smoothened; TZ, transition zone.

Human ciliopathies, including Joubert syndrome (JBTS), arise from cilia dysfunction. The inositol polyphosphate 5-phosphatase INPP5E localizes to cilia and is mutated in JBTS. Murine Inpp5e ablation is embryonically lethal and recapitulates JBTS, including neural tube defects and polydactyly; however, the underlying defects in cilia signaling and the function of INPP5E at cilia are still emerging. We report Inpp5e−/− embryos exhibit aberrant Hedgehog-dependent patterning with reduced Hedgehog signaling. Using mouse genetics, we show increasing Hedgehog signaling via Smoothened M2 expression rescues some Inpp5e−/− ciliopathy phenotypes and “normalizes” Hedgehog signaling. INPP5E’s phosphoinositide substrates PI(4,5)P2 and PI(3,4,5)P3 accumulated at the transition zone (TZ) in Hedgehog-stimulated Inpp5e−/− cells, which was associated with reduced recruitment of TZ scaffolding proteins and reduced Smoothened levels at cilia. Expression of wild-type, but not 5-phosphatase-dead, INPP5E restored TZ molecular organization and Smoothened accumulation at cilia. Therefore, we identify INPP5E as an essential point of convergence between Hedgehog and phosphoinositide signaling at cilia that maintains TZ function and Hedgehog-dependent embryonic development.

Introduction

Primary cilia coordinate several signaling cascades during embryonic development. Cilia are anchored to the plasma membrane by transition fibers that connect the basal body to the plasma membrane, separating the cilia and cytosolic compartments. The intervening region between the basal body and axoneme is termed the transition zone (TZ) and acts a diffusion barrier to contribute to cilia entry and retention mechanisms (Hu et al., 2010; Chih et al., 2011; Williams et al., 2011; Reiter et al., 2012; Szymanska and Johnson, 2012; Jensen et al., 2015). Human ciliopathy syndromes arise from cilia dysfunction and share common phenotypes, including polycystic kidneys, neural tube defects, and polydactyly (Waters and Beales, 2011; Roberson et al., 2015). Growing evidence suggests TZ dysfunction may underlie ciliopathies (Chih et al., 2011; Huang et al., 2011; Sang et al., 2011; Williams et al., 2011; Szymanska and Johnson, 2012; Roberson et al., 2015; Lambacher et al., 2016), although the molecular composition and mechanisms governing TZ function are little characterized.

Vertebrate Hedgehog (Hh) signaling is essential for tissue patterning and embryonic development. Upon Sonic Hedgehog (Shh) ligand binding to Patched (Ptc1), signal transduction is critically dependent on the ciliary accumulation and retention of the transmembrane receptor smoothened (SMO), which in turn modulates Hh-target gene transcription via glioma-associated oncogene homologue-1 (GLI) transcription factors (Corbit et al., 2005; Haycraft et al., 2005; Rohatgi et al., 2007, 2009; Milenkovic et al., 2009; Goetz and Anderson, 2010; Waters and Beales, 2011). However, the mechanisms that govern SMO cilia entry and exit are still emerging. GLI2 and GLI3 predominantly regulate Hh-dependent transcription during development; GLI2 acts primarily as an activator (GLI2A), whereas GLI3 mainly represses transcription after its proteolytic processing to a truncated repressor form (GLI3R; Haycraft et al., 2005; Hui and Angers, 2011). The G protein–coupled receptor GPR161 is a negative regulator of Hh signaling that is recruited to cilia via TULP3 (Tubby-like protein 3) and the IFT-A (intraflagellar transport) complex and promotes GLI3R production (Mukhopadhyay et al., 2013). Recent studies show GPR161 is removed...
from cilia via the accumulation of active SMO at cilia after the induction of Hh signaling (Pal et al., 2016). Consequently, Smo deletion is associated with increased GPR161 levels at cilia (Pal et al., 2016).

Phosphoinositides (PIs) play major roles in regulating many cellular functions, including vesicular trafficking (Ballà, 2013). Recent studies have localized some, but not all, PI species to primary cilia (Vieira et al., 2006; Wei et al., 2008; Franco et al., 2014; Chávez et al., 2015; Garcia-Gonzalo et al., 2015; Jensen et al., 2015; Park et al., 2015); however, their functional role and turnover in response to cilia signaling has not been reported. The inositol polyphosphate 5-phosphatase INPP5E is mutated in the ciliopathies Joubert syndrome (JCTS) and the rarer mental retardation, truncal obesity, retinal dystrophy and microenvis syndrome (Bielas et al., 2009; Jacoby et al., 2009). Ubiquitous deletion of Inpp5e (Inpp5e<sup>−/−</sup>) in mice results in embryonic lethality with a phenotype that recapitulates JBTS, including neural tube defects, polydactyly, and polycystic kidneys (Jacoby et al., 2009). INPP5E degrades phosphatidylinositol(4,5)-bisphosphate (PI(4,5)P<sub>2</sub>) and phosphoinositide 3-kinase (PI3K) signaling (Riobó et al., 2006); however, no studies to date have identified PI(4,5)P<sub>2</sub> and/or PI(3,4,5)P<sub>3</sub> at cilia. Hh signaling activates PI3K signaling (Riobó et al., 2006); however, no studies to date have identified PI(3,4,5)P<sub>3</sub> signals at cilia or examined whether Hh signaling stimulates the turnover of PI(4,5)P<sub>2</sub> and/or PI(3,4,5)P<sub>3</sub> at cilia.

Many INPP5E missense mutations have been identified in JBTS, and all analyzed to date show reduced 5-phosphatase activity toward PI(3,4,5)P<sub>3</sub> and PI(4,5)P<sub>2</sub>, suggesting increased PI(4,5)P<sub>2</sub> and/or PI(3,4,5)P<sub>3</sub> may contribute to abnormal development (Bielas et al., 2009; Travaglini et al., 2013). Importantly, INPP5E localization to cilia is dependent on the growing number of JBTS proteins, such as MKS1, that when mutated or deleted result in the loss of INPP5E cilia localization (Humbert et al., 2012; Thomas et al., 2015; Saal et al., 2016). Thus, cilia mislocalization of INPP5E, and thereby loss of INPP5E function at cilia, is suggested as an important mechanism underlying JBTS. Recent studies demonstrate INPP5E may regulate Hh signaling via modulating the PI(4,5)P<sub>2</sub>-dependent recruitment of GPR161 to cilia (Chávez et al., 2015; Garcia-Gonzalo et al., 2015). However, INPP5E also degrades PI(3,4,5)P<sub>3</sub> and regulates PI3K-dependent cilia stability. INPP5E-mediated degradation of PI3K-generated PI(3,4,5)P<sub>3</sub> is essential to cilia function (Kisseleva et al., 2002; Jacoby et al., 2009) by yet-to-be-identified mechanisms. In addition, recent studies have shown increased PI3K–AKT–mTOR signaling drives polycystic kidney disease in kidney-tubule–specific Inpp5e knockout mice (Hakim et al., 2016).

Here, we show Inpp5e<sup>−/−</sup> mice exhibit abnormal Hh-dependent patterning in vivo and reduced cilia accumulation of the Hh signaling components SMO and GLI2, leading to decreased Hh signaling. Proof-of-principle mouse genetic studies demonstrate Inpp5e null ciliopathy phenotypes are in part rescued by constitutive hyperactivation of Hh signaling via SMOM2 expression. At the TZ, PI(3,4,5)P<sub>3</sub> signals increase in response to Hh signaling and further increase with Inpp5e deletion, leading to changes in the molecular organization and function of the TZ and reduced accumulation of SMO at cilia. Therefore, our study identifies INPP5E as a novel regulator of TZ function that integrates PI3K and Hh signaling at cilia.

**Results**

**Murine deletion of Inpp5e results in disordered Hh-dependent patterning and decreased high-level Hh signaling during embryonic development**

Recently, INPP5E has been shown to modulate the trafficking of the Hh pathway suppressor GPR161 to cilia and regulate Hh signaling in cultured cells (Chávez et al., 2015; Garcia-Gonzalo et al., 2015). To investigate whether in vivo Hh signaling/patterning is perturbed with Inpp5e ablation, we deleted the 5-phosphatase by mating a conditionally modified mouse (floxing exons 2 to 6, Inpp5e<sup>tm1.Cmit</sup>; Fig. 1 a), with a germ-line Cre-deletor strain (CMV-Cre, hereafter Inpp5e<sup>−/−</sup>). Inpp5e deletion in primary mouse embryonic fibroblasts (pMEFs) was confirmed by quantitative RT-PCR (Fig. S1 b). Inpp5e<sup>−/−</sup> embryos died at ~E18.5, when polycystic kidneys (100%, n = 26/26) were observed (Fig. S1 c). From midgestation, Inpp5e<sup>−/−</sup> embryos showed multiple abnormalities consistent with cilia dysfunction and/or aberrant Hh signaling, including bilateral anophthalmos (100%, n = 26/26) in the presence or absence of exencephaly (65%, n = 17/26; Fig. 1 a and b), cleft palate (76%, n = 20/26; Figs. 1 c and S1 d), and single-digit preaxial hindlimb polydactyly (100%, n = 26/26; Fig. 1 d). These defects are consistent with a previous study of Inpp5e<sup>−/−</sup> embryos, but in addition, we identify edema (69%, n = 18/26; Fig. 1 a and b, arrows), delayed and reduced vertebral body ossification (100%, n = 4/4; Figs. 1 e and S1 e), reduced and absent ossification of the occipital bone (50%, n = 2/4; Fig. S1 f) and 13th rib (75%, n = 3/4; Figs. 1 f and S1 g), respectively, and pulmonary hypoplasia coupled with left lung isomerization (100%, n = 6/6; Fig. 1 g). This phenotypic spectrum broadly phenocopies INPP5E mutations in JBTS (Bielas et al., 2009; Jacoby et al., 2009).

To assess the contribution of INPP5E to Hh signaling in vivo, we examined the patterning of neuroepithelial cells in the developing neural tube of Inpp5e<sup>−/−</sup> embryos which is highly dependent on the fine balance of GLI activator and repressor function. Shh secreted from the notochord induces the floor plate and specifies motor neurons and various classes of interneurons across the dorsoventral axis in a concentration-dependent manner. Induction of the ventral floor plate requires the highest level of Hh signaling and is primarily dependent on GLI2 activator (GLI2A) function, whereas GLI3R in the dorsal neural tube defines the dorsal extent of intermediate neuroepigeneiters (Ding et al., 1998; Matise et al., 1998; Litingtung and Chiang, 2000). At embryonic day 10.5 (E10.5), Shh expression was abrogated (Fig. 1 h) and cell morphology was altered (Fig. 1 j, arrowheads) in the Inpp5e<sup>−/−</sup> floor-plate region, suggesting cells requiring the highest levels of Shh were incorrectly specified. Islet1-positive motor neuron progenitors were detected ectopically in the midline and expanded dorsally in Inpp5e<sup>−/−</sup> embryos (Fig. 1 i and j). Nkx2.2-positive P3 interneuron progenitors were mislocalized in the ventral midline (Fig. 1 k), and Pax6-positive P2 interneuron progenitors shifted dorsally in Inpp5e<sup>−/−</sup> embryos (Fig. 1 l). Quantification revealed an increased area and dorsal expansion of Pax6-positive progenitors in the Inpp5e<sup>−/−</sup> neural tube but no change in ventral positioning (Fig. 1 m). Interestingly, the neural tube patterning in Inpp5e<sup>−/−</sup> embryos is similar to mice with mutations in Arl13b, which is also mutated in JBTS, and Arl13b is required for INPP5E cilia localization (Caspari et al., 2007; Humbert et al., 2012). In both Arl13b and Inpp5e<sup>−/−</sup>
mutant mice, floor plate cells that require the highest level of Hh signaling are not specified, and cells dependent on intermediate Hh levels are expanded. Defects in GLI activator are proposed to underlie the abnormal neural tube patterning in Arl13b mutant mice, whereas GLI3 repressor was unaffected (Caspy et al., 2007). The similarities between the Arl13b and Inpp5e mutants suggest defective GLI activator may also contribute to the disordered neural tube patterning with Inpp5e deletion. However, Inpp5e ablation has recently been shown to promote GPR161 cilia accumulation. GPR161 negatively regulates Hh signaling via increasing cyclic AMP levels and thereby promoting GLI3 repressor formation (Mukhopadhyay et al., 2013; Chávez et al., 2015; Garcia-Gonzalo et al., 2015). Indeed, GPR161 is highly expressed in the developing neural tube, and its deletion in mice gives rise to a ventralized neural tube phenotype similar to IFT-A mutants (Matuson et al., 2008; Mukhopadhyay et al., 2013). Thus, although the comparative phenotype analysis suggests defective GLI activator contributes to the Inpp5e−/− neural tube phenotype, we cannot exclude a role for GLI3 repressor as predicted by the recently described INPP5E–GPR161–GLI3...
Enhanced Hh signaling via SMO activation rescues Inpp5e−/− mouse phenotypes

The contribution of decreased Hh signaling to the Inpp5e null phenotype was assessed using a mouse genetics approach. SMOM2 (Trp535Leu) is a constitutively active SMO mutant originally identified in basal cell carcinoma that localizes to cilia independent of the Shh ligand (Xie et al., 1998; Jung et al., 2016). Expression of SMOM2 in mice or in cultured cells results in the hyperactivation of Hh signaling (Corbit et al., 2005; Mao et al., 2006; Wong et al., 2009). Therefore, we reasoned that SMOM2 expression in the context of Inpp5e deletion may in part normalize Hh signaling. To this end, Inpp5e null mice expressing constitutively active SMO (SMOM2) were generated (Inpp5e−/−;CMVCre;SmoM2). Analysis of E15.5 Inpp5e−/−;CMVCre;SmoM2 embryos revealed a striking “rescue” of phenotypes commonly observed in Inpp5e null (Inpp5e−/−;CMVCre) single-mutant mice, including complete rescue of the bilateral anophthalmia and exencephaly (Table S1 and Fig. 2, a and b) and partial rescue of the edema (Table S1 and Fig. 2 b), hindlimb polydactyly (Table S1 and Fig. 2 c), and cleft palate (Table S1). In contrast to both the Inpp5e−/−;CMVCre and Inpp5e−/−;CMVCre;SmoM2 single-mutant mice that show bilateral anophthalmia, formation of the lens, retina, and optic nerve appeared relatively normal in Inpp5e−/−;CMVCre;SmoM2 embryos (Fig. 2 e), although one embryo (of eight analyzed) exhibited coloboma (Fig. S2 a). Significantly, at E15.5, the previously unreported complex dysmorphology associated with constitutive SMOM2 signaling during embryonic development (Inpp5e−/−;CMVCre;SmoM2), including the midline expansion (Fig. 2 a, double arrow), fore- and hindlimb polydactyly (Table S1 and Fig. 2, c and d), bilateral anophthalmia (Table S1 and Fig. 2, a and e), and loss of neural tube architecture (Table S1 and Fig. 2 b and c), was also rescued by Inpp5e−/−;CMVCre;SmoM2 embryos.

Inpp5e deletion reduces Hh-stimulated cilia accumulation of SMO and GLI2

The cilia accumulation and subsequent activation of SMO after Hh stimulation is critical for activating downstream signaling (Corbit et al., 2005). We investigated the cilia localization of Hh pathway components in pMEFs before and after Hh pathway stimulation using SAG, conditions under which SMO accumulates at cilia (Chen et al., 2002; Rohatgi et al., 2007; Wang et al., 2009; Wu et al., 2012). Endogenous SMO was virtually undetected at cilia in nonstimulated Inpp5e−/− and Inpp5e−/− pMEFs when compared with Inpp5e+/+ pMEFs, consistent with attenuation of Hh signaling. Therefore, Inpp5e−/− embryos and pMEFs show evidence of reduced Hh signaling and normal cilia number, suggesting that the coordination of Hh signaling at cilia may be disrupted with Inpp5e deletion.
In the absence of Hh activation, GLI2 localizes at the cilia tip at low levels. SMO stimulation promotes GLI2 trafficking and its increased accumulation at the cilia tip, which is causally linked to the cilia enrichment of SMO (Chen et al., 2009; Kim et al., 2009). GLI2 was detected at low levels at cilia in untreated Inpp5e+/− and Inpp5e−/− pMEFs (unpublished data). After SAG treatment, GLI2 accumulated at the cilia tip immediately distal the axoneme in Inpp5e+/− pMEFs (Fig. 3 c); however, in Inpp5e−/− pMEFs, GLI2 was more evenly distributed along the axoneme and showed reduced accumulation at the cilia tip in response to SAG (Fig. 3 c), an observation confirmed by cilia tip GLI2 MFI analysis (Fig. 3 c). Together, these findings reveal the cilia accumulation and cilia tip localization of the Hh signal transducers SMO and GLI2, respectively, are reduced in response to Hh pathway activation with Inpp5e deletion.

SMO regulates GPR161 levels at cilia by promoting its removal from cilia after Hh pathway activation (Pal et al., 2016). Endogenous GPR161 cilia levels were increased in untreated and SAG-treated Inpp5e+/− pMEFs compared with wild-type as described (Fig. 3 d; Garcia-Gonzalo et al., 2015). The reduced accumulation of SMO at cilia in Inpp5e−/− pMEFs after Hh pathway activation may contribute to the increased level of GPR161. In addition, GPR161 cilia levels were increased in untreated rescue Inpp5e+/−;CMVCre;SmoM2 pMEFs compared with wild-type (Fig. 3 d). These studies show that despite the rescue of some Inpp5e−/− phenotypes by concomitant SMOM2 expression, GPR161 is abnormally retained at cilia. Therefore, the regulation of GPR161 by INPP5E is not the only mechanism by which the 5-phosphatase regulates Hh signaling, and the partial rescue of Inpp5e+/−;CMVCre;SmoM2 is not caused by the relocalization of GPR161 away from cilia.

To evaluate INPP5E function in regulating cilia membrane composition the localization of cilia-localized receptors not involved in or spatially regulated by Hh signaling was investigated. Endogenous polycystin II (Fig. 3 e) and recombinant FLAG-tagged HTR6 (Fig. S3 c) localized to cilia in untreated Inpp5e+/− and Inpp5e−/− pMEFs. However, after SAG treatment, both receptors showed reduced cilia localization in Inpp5e−/− pMEFs compared with wild-type cells (Figs. 3 e and S3 c), also shown by polycystin II cilia MFI analysis (Fig. 3 e). These findings are consistent with the reported mislocalization of INPP5E in cell culture and in vivo models, both of which will be discussed in the next section.
The MFI of polycystin II at cilia was measured in untreated (+SAG) and SAG-treated (+SAG) Inpp5e+/+ with polycystin II and act-tubulin antibodies and DAPI and imaged using confocal microscopy. Arrow indicates cilia. Bars, 500 nm. The MFI of polycystin II at cilia was measured in untreated (+SAG) and SAG-treated (+SAG) Inpp5e+/+ and Inpp5e−/− pMEFs. Bars represent mean ± SEM. n = 3 pMEF lines per genotype, >100 cells per genotype; **, P < 0.001; ****, P < 0.0001. (b) HA-INPP5E (HA-INPP5E), or phosphatase-dead HA-INPP5ED480N (HA-D480N) were SAG treated (+SAG), fixed, permeabilized, and costained with DAPI, and GPR161 and act-tubulin antibodies and imaged using confocal microscopy. Bars, 500 nm. The MFI of GPR161 at the cilia axoneme was measured. Bar represents mean ± SEM, n = 3 pMEF lines per genotype, >90 cells per genotype, >90 cells per genotype; **, P < 0.01; ****, P < 0.0001. (c) Inpp5e−/− medium (−/−) pMEFs transiently expressing either HA vector [not depicted], wild-type HA-INPP5E (HA-INPP5E), or phosphatase-dead HA-INPP5ED480N (HA-D480N) were SAG treated (+SAG), fixed, permeabilized, and costained with SMO, act-tubulin, and HA antibodies (HA staining not depicted) and imaged using confocal microscopy. The proximal and distal end of the cilium is orientated left to right, respectively. Bars, 500 nm. (right graph) The relative Ptch1 transcript level was determined and normalized to Inpp5e−/−. Transfection efficiency of HA vector was 81% ± 2.08% (SEM). Bars represent mean ± SEM. n = 3 pMEF lines per genotype, >90 cells per genotype; **, P < 0.01; ****, P < 0.0001.
of PKD-2 in cili-1 mutants, the closest Caenorhabditis elegans homologue of INPP5E (Bae et al., 2009). Also, although another recent study reports no change in polycystin II cilia localization with loss of Inpp5e (Garcia-Gonzalo et al., 2015), this study did not examine receptor localization after Hh pathway activation, the specific conditions under which the reduced polycystin II cilia localization was observed here in Inpp5e−/− pMEFs. We were unable to confidently measure FLAG-HTR6 intensity at cilia, as its expression in Inpp5e−/− pMEFs impacted ciliogenesis; however, reduced cilia enrichment of the receptor was observed in some ciliated cells with loss of Inpp5e (Fig. S3 c). We also evaluated the barrier function of the TZ to prevent the cilia entry of nonciliary proteins (Fig. S3 d). CEACAM-1 is a highly mobile plasma membrane glycoprotein that ectopically localizes to cilia in some models with loss of TZ barrier function (Chih et al., 2011; Francis et al., 2011). Recombinant GFP–CEA was not detected at cilia in either Inpp5e−/− or Inpp5e−/− pMEFs irrespective of AGP treatment, suggesting Inpp5e deletion does not compromise the cilia entry barrier function of the TZ (Fig. S3 d). Collectively, these findings reveal loss of Inpp5e reduces the cilia accumulation of some cilia receptors specifically after Hh pathway activation via AGP but that cilia entry mechanisms remain intact.

**INPP5E regulates phosphoinositide signals at the cilia TZ**

The activation of Hh signaling in cultured cells promotes PI3K-dependent phosphorylation of AKT, suggesting pathway crosstalk (Riobó et al., 2006). However, whether Hh-mediated activation of PI3K, which in turn generates PI(3,4,5)P3, occurs locally at cilia is unknown. To this end, PI(4,5)P2 and PI(3,4,5)P3 cilia localization and their regulation by INPP5E and AGP signaling were examined using established immunocytochemical techniques that preserve organelle membranes and validated antibodies specific to PI(4,5)P2, or PI(3,4,5)P3 (Hammond et al., 2006; Hop et al., 2008). PI(4,5)P2 localizes to the proximal end of cilia in SV40-transformed MEFs, C. elegans, and Drosophila melanogaster; however, the discrete distribution of PI(4,5)P2 to cilia subdomains via colocalization with cilia domain markers has not been reported (Garcia-Gonzalo et al., 2015; Jensen et al., 2015; Park et al., 2015). Here, PI(4,5)P2 was enriched proximal to the axoneme at the cilia base in untreated Inpp5e−/− pMEFs and was also distributed at low levels along the axoneme and colocalized with ac-tubulin (Figs. 4 a and S4 a). At the cilia base, PI(4,5)P2 is detectably localized between ac-tubulin and pericentrin (Fig. S4 b and showed a similar distribution in hTERT retinal pigment epithelial (RPE) cells (Fig. S4 c, top), where it colocalized with the TCR at TCTN1 (Fig. S4 c, middle; Garcia-Gonzalo et al., 2011). Notably colocalization of HA-INPP5E with TCTN1 revealed that in addition to localizing to the cilia axoneme, the 5-phosphatase partially localizes to the TZ in a subset of cells thereby in proximity to its substrates PI(4,5)P2 (Fig. S4 c, bottom). At steady state, Inpp5e−/− pMEFs showed a PI(4,5)P2 distribution similar to wild type (Figs. 4 a and S4 a), with a comparable signal intensity (MFI) at the TZ (Fig. 4 b) and PI(4,5)P2 to ac-tubulin ratio (Fig. S4 d). However, at the axoneme, PI(4,5)P2 intensity was modestly increased with Inpp5e deletion, consistent with previous studies (Fig. 4 b; Garcia-Gonzalo et al., 2015). In control studies, treatment of Inpp5e−/− pMEFs after cell fixation with neomycin, which binds and sequesters this PI(4,5)P2 (Liscovitch et al., 1991), attenuated cilia-associated PI(4,5)P2 signals (Fig. S4 e). In nonciliated cells after growth factor stimulation, plasma membrane PI(4,5)P2 signals decrease because of its cleavage by phospholipase C and/or PI3K-mediated phosphorylation of PI(4,5)P2, generating PI(3,4,5)P3; however, the dynamics of PI(4,5)P2 at cilia in response to agonist stimulation has not been reported (Ballà, 2013). After AGP treatment of Inpp5e−/− pMEFs the number of PI(4,5)P2-positive cilia (Fig. 4 c) and the MFI of PI(4,5)P2 at the TZ was reduced, but axoneme PI(4,5)P2 intensity slightly increased (Fig. 4 b). As an additional readout of the relative TZ PI(4,5)P2 signal per cilia, the PI(4,5)P2/ac-tubulin ratio was measured and was also reduced compared with untreated wild-type pMEFs (Fig. S4 d). Therefore, PI(4,5)P2 signals decrease at the TZ but increase along the axoneme after AGP treatment of Inpp5e−/− pMEFs. The number of PI(4,5)P2-positive cilia was unchanged after AGP treatment of Inpp5e−/− pMEFs (Fig. 4 c). PI(4,5)P2 intensity at the TZ decreased in Inpp5e−/− pMEFs after AGP treatment but was increased relative to wild-type (Fig. 4, a and b; and Fig. S4 d). In addition AGP treatment of Inpp5e−/− pMEFs resulted in a loss of axoneme PI(4,5)P2 signals (Fig. 4, a and b; and Fig. S4 a). Therefore with Inpp5e deletion PI(4,5)P2 signals are increased after activation of Hh signaling.

INPP5E is a potent PI(3,4,5)P3 5-phosphatase in vitro and in vivo (Kisseleva et al., 2000; Hakim et al., 2016) but PI(3,4,5)P3 has not been identified at cilia. Endogenous PI(3,4,5)P3 and ac-tubulin containing revealed that ~50% of Inpp5e−/− pMEFs showed PI(3,4,5)P3 at the cilia base (Fig. 4, d and e; and Fig. S4, f and g). PI(3,4,5)P3 also localized to the cilia base in RPE cells (Fig. S4 h, top), with an intervening distribution between the axoneme and pericentrin (Fig. S4 g). In addition, PI(3,4,5)P3 colocalized with the TZ marker, TCTN1 (Fig. S4 h, bottom), analogous to our findings for PI(4,5)P2 (Fig. S4 c, bottom). The number of PI(3,4,5)P3-positive cilia was increased in Inpp5e null pMEFs (Fig. 4 e), as was the TZ PI(3,4,5)P3 intensity (Fig. 4 f) and TZ PI(3,4,5)P3/ac-tubulin ratio (Fig. S4 i) relative to wild-type cells. After AGP treatment of Inpp5e−/− and Inpp5e−/− pMEFs, PI(3,4,5)P3 was detected at the TZ (Fig. 4 d) of all cilia (Fig. 4 e), and the TZ PI(3,4,5)P3 intensity (Fig. 4 f) and TZ PI(3,4,5)P3/ac-tubulin ratio were increased in both genotypes compared with their respective untreated controls (Fig. S4 i) and were significantly higher in Inpp5e null cells (Figs. 4 f and S4 i). Therefore, activation of Hh signaling increases PI(3,4,5)P3 signals at the TZ in wild-type and Inpp5e−/− pMEFs, and the relative PI(3,4,5)P3 signal intensity was further increased with Inpp5e deletion (Figs. 4 f and S4 i). In control studies, PI3K inhibitor (LY294003) treatment reduced PI(3,4,5)P3 signals at the cilia base in AGP-stimulated Inpp5e−/− pMEFs (Fig. S4 j). Additionally the fidelity of PI(3,4,5)P3 and PI(4,5)P2 immunostaining was further confirmed by colocalizing the endogenous lipid species with transfected GFP-Btk or GFP-PLC biosensors, which detect PI(3,4,5)P3 and PI(4,5)P2, respectively (Ballà, 2013; Fig. S4, k and l). In summary, these findings show that INPP5E partially localizes to the TZ in proximity to its substrates, and with Inpp5e deletion, both PI(4,5)P2 and PI(3,4,5)P3 signals are increased after Hh pathway activation. A schematic of the relative PI(4,5)P2 and PI(3,4,5)P3 signal intensity at the TZ of Inpp5e−/− and Inpp5e−/− pMEFs in the presence or absence of AGP is shown in Fig. S4 m. Collectively, this analysis reveals that PI(4,5)P2 and PI(3,4,5)P3 signals are enriched at the TZ and are differentially and dynamically regulated by Hh signaling and INPP5E.
INPP5E regulates the molecular organization of the cilium TZ

Emerging evidence suggests a subset of ciliopathies, including Meckel–Gruber and JBTS, arise from dysfunction of the TZ, resulting in aberrant cilia signaling (Hu et al., 2010; Chih et al., 2011; Garcia-Gonzalo et al., 2011; Williams et al., 2011). Several ciliopathy proteins such as MKS1, B9D proteins, and Tectonic module components localize to the TZ and modulate TZ function via scaffold assembly (Chih et al., 2011; Garcia-Gonzalo et al., 2011; Sang et al., 2011; Williams et al., 2011). The TZ is essential for cilia compartmentalization and regulates the ciliary membrane composition of signaling receptors via their localization to the TZ. Therefore, we examined whether increased PI(4,5)P2 and PI(3,4,5)P3 signals at the TZ of Hh-stimulated Inpp5e−/− pMEFs were associated with mislocalization of TZ scaffolding proteins.

ARL13B is mutated in JBTS and is necessary for INPP5E cilia localization (Humbert et al., 2012); however, whether INPP5E plays a role in ARL13B cilia localization is unknown. ARL13B localizes along the axoneme; however, in MKS1 or B9, complex reduction-of-function mutants with reduced TZ function, ARL13B is partially redirected to the TZ (Cevik et al., 2013). In both untreated and SAG-treated Inpp5e−/− pMEFs, either untreated (−SAG) or SAG treated (+SAG) were costained with PI(3,4,5)P3 and acetylated α-tubulin antibodies as in panel d. The MFI of PI(3,4,5)P3 at the cilia “transition zone” or “axoneme” was measured. Bars represent mean ± SEM. n = 3 pMEF lines per genotype, ~60 cells per genotype; **, P < 0.01; *, P < 0.05; ***, P < 0.005; ****, P < 0.0001. (f) Inpp5e−/− (+/+ and +/+) pMEFs, either untreated (−SAG) or SAG treated (+SAG) were costained with PI(4,5)P2 and acetylated α-tubulin antibodies as in panel d. The MFI of PI(4,5)P2 at the cilia “transition zone” or “axoneme” was measured. Bars represent mean ± SEM. n = 3 pMEF lines per genotype, ~60 cells per genotype; *, P < 0.05; **, P < 0.01; ***, P < 0.005. AU, arbitrary units.

Figure 4. PI(4,5)P2 and PI(3,4,5)P3 localize to the cilia TZ and are regulated by INPP5E and Hh signaling. (a) Inpp5e−/− (+/+) and Inpp5e−/− (−/−) pMEFs, either untreated (−SAG) or SAG treated (+SAG), were fixed, permeabilized, and costained with PI(4,5)P2 and acetylated α-tubulin antibodies and imaged using confocal microscopy. The proximal and distal end of the cilium is orientated left to right, respectively. Bars, 500 nm. (b) Inpp5e−/− (+/+) and Inpp5e−/− (−/−) pMEFs, either untreated (−SAG) or SAG treated (+SAG), were costained with PI(4,5)P2 and acetylated α-tubulin antibodies as in panel a. The MFI of PI(4,5)P2 at the cilia “transition zone” or “axoneme” was measured. Bars represent mean ± SEM. n = 3 pMEF lines per genotype, ~60 cells per genotype; *, P < 0.05; **, P < 0.01; ***, P < 0.005; ****, P < 0.0001. (c) Inpp5e−/− (+/+) and Inpp5e−/− (−/−) pMEFs, either untreated (−SAG) or SAG treated (+SAG), were costained with PI(4,5)P2 and acetylated α-tubulin antibodies as in panel a. The percentage of acetylated α-tubulin–positive cilia that were also positive for PI(4,5)P2 was scored. Bars represent mean ± SEM. n = 3 pMEF lines/genotype, ~60 cells per genotype; ***, P < 0.001; ****, P < 0.0001. (d) Inpp5e−/− (+/+) and Inpp5e−/− (−/−) pMEFs, either untreated (−SAG) or SAG treated (+SAG), were fixed, permeabilized, and costained with PI(3,4,5)P3 and acetylated α-tubulin antibodies and imaged using confocal microscopy. The proximal and distal end of the cilium is orientated left to right, respectively. Bars, 500 nm. (e) Inpp5e−/− (+/+) and Inpp5e−/− (−/−) pMEFs either untreated (−SAG) or SAG treated (+SAG) were costained with PI(3,4,5)P3 and acetylated α-tubulin antibodies as in panel d. The percentage of acetylated α-tubulin–positive cilia that were also positive for PI(3,4,5)P3 was scored. Bars represent mean ± SEM. n = 3 pMEF lines per genotype; ***, P < 0.001; ****, P < 0.0001. Inpp5e−/− (+/+) and Inpp5e−/− (−/−) pMEFs, either untreated (−SAG) or SAG treated (+SAG), were associated with mislocalization of TZ scaffolding proteins after Hh pathway activation (Fig. 5 a arrow).
INPP5E regulates the molecular organization of the cilia TZ. (a) Inpp5e+/+ and Inpp5e−/− pMEFs either untreated (−SAG) or SAG treated (+SAG) were fixed, permeabilized and costained with ARL13B and acetylated α-tubulin (ac-tubulin) antibodies and imaged using confocal microscopy. The proximal and distal end of the cilium is orientated left to right, respectively. Bar, 500 nm. Arrow indicates ectopic ARL13B localization. The MFI of ARL13B at the transition zone (TZ MFI) was measured. Bars represent mean ± SEM. n = 3 pMEF lines per genotype, ~60 cells per genotype; *, P < 0.05. (b) Inpp5e+/+ and Inpp5e−/− pMEFs, either untreated (−SAG) or SAG treated (+SAG), were fixed, permeabilized, and costained with MKS1 and acetylated α-tubulin (ac-tubulin) antibodies and imaged using confocal microscopy. The proximal and distal end of the cilium is orientated left to right, respectively. Bar, 500 nm. The MFI of the respective TZ component at the TZ was measured. Bars represent mean ± SEM. n = 3 pMEF lines per genotype, ~60 cells per genotype; **, P < 0.01. (c) Inpp5e+/+ and Inpp5e−/− pMEFs transfected with Emerald-MKS1 were either untreated (−SAG) or SAG treated (+SAG), fixed, permeabilized, stained with ac-tubulin antibodies, and imaged using confocal microscopy. The proximal and distal end of the cilium is orientated left to right, respectively. Bar, 500 nm. The MFI of Emerald-MKS1 at the TZ was measured (TZ MFI). Bars represent mean ± SEM. n = 3 pMEF lines per genotype, ~60 cells per genotype; **, P < 0.01. (d–f) Inpp5e+/+ and Inpp5e−/− pMEFs, either untreated (−SAG) or SAG treated (+SAG), were fixed, permeabilized, and costained with TCTN1 (d), TMEM231 (e), or B9D1 (f) and ac-tubulin and imaged using confocal microscopy. The proximal and distal end of the cilium is orientated left to right, respectively. Bar, 500 nm. The MFI of the respective TZ component at the transition zone (TZ MFI) was measured. Bars represent mean ± SEM. n = 3 pMEF lines per genotype, ~60 cells per genotype; *, P < 0.05; **, P < 0.01. AU, arbitrary units.

To assess TZ composition with Inpp5e deletion, the localization of several TZ components was evaluated at steady state (−SAG) and after Hh pathway activation via SAG (+SAG; Fig. 5, b–f). The Tectonic TZ component TCTN1 interacts with MKS1 and B9D1. TCTN1 is required for the TZ localization of B9D1 and TMEM231 and together with other TZ components cooperatively regulates TZ function (Chih et al., 2011; Garcia-Gonzalo et al., 2011; Williams et al., 2011; Szymanska and Johnson, 2012). Endogenous MKS1 (and recombinant Emerald-MKS1), TCTN1, TMEM231, and B9D1 localized to the TZ at steady state in Inpp5e+/+ and Inpp5e−/− pMEFs and in SAG-treated Inpp5e+/+ pMEFs but showed reduced or absent TZ localization in SAG-treated Inpp5e−/− pMEFs, consistent with the contention that Inpp5e ablation impairs the TZ localization of scaffolding proteins after Hh pathway activation (Fig. 5, b–f).

In control studies endogenous MKS1, TCTN1, B9D1, and TMEM231 colocalized with recombinant MKS1 (Fig. S5 a). Septins form a diffusion barrier in budding yeast and in mammalian cells play a scaffolding role (Mostowy and Cossart, 2012). At cilia, septins form a SEPT2–SEPT5–SEPT7 complex and localize at the cilia base and along the axoneme, where they regulate ciliogenesis and cilia length (Ghossoub et al., 2013). At the cilia base, septins are proposed to form a diffusion barrier separating the cilia and plasma membranes, and SEPT2 facilitates the cilia retention of receptors, including SMO and HTR6, after Hh pathway activation (Hu et al., 2010). In addition, SEPT2 is required for the TZ localization of some B9 complex components (Garcia-Gonzalo et al., 2011). The mechanisms mediating the cilia localization of septins is unknown; however, septins interact with several PIs, including the INPP5E substrates PI(4,5)P2 and PI(3,4,5)P3 (Mostowy and Cossart, 2012). The role of PIs in septin function is complex, as various PIs regulate septin localization and oligomerization, often in a concentration-dependent manner. Some studies suggest...
septins preferentially bind and assemble on membranes with low PI(4,5)P2, whereas other studies propose high PI(4,5)P2 is preferred (Maléth et al., 2014; Badrane et al., 2016). In steady-state wild-type and Inpp5e−/− pMEFs, SEPT2 localized at the axoneme and cilia base (Fig. S5 b, arrows), as reported previously (Hu et al., 2010; Ghossoub et al., 2013). SEPT2 cilia distribution was largely unchanged in Inpp5e+/+ pMEFs after SAG stimulation; however, in SAG-treated Inpp5e−/− pMEFs, SEPT2 localization at the cilia base was reduced, with only low level SEPT2 puncta at the axoneme observed (Fig. S5 b). Collectively, the mislocalization of TCTN1, MKS1, TMEM231, B9D1, and SEPT2 from the TZ in Inpp5e null cells after Hh pathway activation is consistent with a compromised TZ.

To determine whether the INPP5E-mediated degradation of PI(4,5)P2 and PI(3,4,5)P3 was critical for the normal assembly of scaffolding proteins at the TZ in the response to Hh signaling, Inpp5e−/− pMEFs were transiently transfected with HA vector (top), wild-type HA-INPP5E (HA-INPP5E, second panel), phosphatase-dead HA-INPP5ED480N (HA-D480N, third panel) or mock transfected and treated with LY294003 (PI3-kinase inhibitor, fourth panel) were SAG treated (+SAG), fixed, permeabilized, and stained with TCTN1 (a), TMEM231 (b), or B9D1 (c) antibodies and acetylated α-tubulin (ac-tubulin) and HA antibodies (HA staining to detect transfected cells is not depicted) and imaged using confocal microscopy. The proximal and distal ends of the cilia are orientated left to right, respectively. Arrow indicates TZ localization. Bars, 500 nm. The MFI of TCTN1 (a), TMEM231 (b), or B9D1 (c) was measured in Inpp5e−/− pMEFs expressing HA vector (black bars), HA-INPP5E (light gray bars), HA-D480N (dark gray bars), and mock transfected with LY294003 treatment (white bars). Bars represent mean ± SEM. n = 3 transfections, ∼50 cells per condition; *, P < 0.05. (d) Inpp5e−/− pMEFs transiently expressing HA vector (top), wild-type HA-INPP5E (HA-INPP5E, second panel), or phosphatase-dead HA-INPP5ED480N (HA-D480N, third panel) were SAG treated (+SAG), fixed, permeabilized, and stained with SEPT2, ac-tubulin and HA antibodies (HA staining to detect transfected cells is not depicted) and imaged using confocal microscopy. The proximal and distal ends of the cilia are orientated left to right, respectively. Arrow indicates cilia base. Bars, 500 nm. AU, arbitrary units.

INPP5E 5-phosphatase activity regulates the molecular organization of the cilia TZ. (a–c) Inpp5e−/− pMEFs transiently expressing HA vector (top), wild-type HA-INPP5E (HA-INPP5E, second panel), phosphatase-dead HA-INPP5ED480N (HA-D480N, third panel) or mock transfected and treated with LY294003 (PI3-kinase inhibitor, fourth panel) were SAG treated (+SAG), fixed, permeabilized, and stained with TCTN1 (a), TMEM231 (b), or B9D1 (c) antibodies and acetylated α-tubulin (ac-tubulin) and HA antibodies (HA staining to detect transfected cells is not depicted) and imaged using confocal microscopy. The proximal and distal ends of the cilia are orientated left to right, respectively. Arrow indicates TZ localization. Bars, 500 nm. The MFI of TCTN1 (a), TMEM231 (b), or B9D1 (c) at the transition zone (TZ MFI) was measured in Inpp5e−/− pMEFs expressing HA vector (black bars), HA-INPP5E (light gray bars), HA-D480N (dark gray bars), and mock transfected with LY294003 treatment (white bars). Bars represent mean ± SEM. n = 3 transfections, ∼50 cells per condition; *, P < 0.05. (d) Inpp5e−/− pMEFs transiently expressing HA vector (top), wild-type HA-INPP5E (HA-INPP5E, second panel), or phosphatase-dead HA-INPP5ED480N (HA-D480N, third panel) were SAG treated (+SAG), fixed, permeabilized, and stained with SEPT2, ac-tubulin and HA antibodies (HA staining to detect transfected cells is not depicted) and imaged using confocal microscopy. The proximal and distal ends of the cilia are orientated left to right, respectively. Arrow indicates cilia base. Bars, 500 nm. AU, arbitrary units.

INPP5E regulates the function of the cilia TZ

The TZ has various functions, including cilia and cytosol compartmentalization via excluding the cilia entry of noncilia proteins. The TZ also acts to retain cilia receptors after Hh pathway activation to allow cilia receptor accumulation (Hu et al., 2010; Chih et al., 2011). The function of the TZ can be assessed experimentally by live-cell imaging of cilia-localized receptors after photobleaching (Hu et al., 2010; Chih et al., 2011). Knockdown of TZ components, including B9d1 and Tmem231...
or Sept2 depletion, have shown TZ dysfunction experimentally by increased FRAP of ciliary receptors compared with controls after Hh pathway activation (Hu et al., 2010; Chih et al., 2011). To investigate the regulation of SMO dynamics at cilia by INPP5E, Inpp5e+/+ and Inpp5e−/− pMEFs were transiently expressing YFP-SMO to allow for detection of fluorescently labeled SMO in live cells stimulated with SAG to activate Hh signaling and thereby induce YFP-SMO accumulation at cilia. Recombinant YFP-SMO showed reduced cilia localization in ∼50% of Inpp5e−/− pMEFs (unpublished data); however, for the FRAP analysis, Inpp5e−/− cells with YFP-SMO cilia intensities similar to wild type were selected (Fig. 7 a). The recovery of YFP-SMO at cilia was assessed in SAG-treated Inpp5e+/+ versus Inpp5e−/− pMEFs via FRAP, which measures the bidirectional movement of the receptor through the TZ (Fig. 7). At time 0 min, ∼90% of the ciliary YFP-SMO signal (Fig. 7 a, boxed regions in inset) was bleached, with 10% of the YFP-SMO signal retained to allow Z-position tracking of the cilium. YFP-SMO recovery was measured for 400 s after photobleaching and the cilia fluorescence to cytoplasmic fluorescence ratio (Fcilia/c) determined over time (Fig. 7 b). Data were expressed as fractional recovery of Fcilia/c, where each postbleach time point was divided by the mean prebleach Fcilia/c value and then normalized to time 0 min (see Materials and methods). The FRAP of YFP-SMO was comparable between genotypes for the first 100 s, after which time recovery plateaued in Inpp5e+/+ pMEFs (Fig. 7, a and b).

However, in Inpp5e−/− pMEFs, YFP-SMO FRAP increased over time compared with Inpp5e+/+, suggesting ongoing and continued exchange of the YFP-SMO ciliary signal consistent with a loss in the ciliary retention of YFP-SMO (Hu et al., 2010). These studies reveal INPP5E is essential for TZ function after Hh pathway activation (Fig. 7, a and b).

Discussion

Ciliopathies arise because of cilia dysfunction and aberrant cilia-mediated signal transduction (Waters and Beales, 2011). INPP5E is mutated in JBTS, and its ablation in mice leads to ciliopathy phenotypes by molecular mechanisms that are still emerging (Jacoby et al., 2009; Chávez et al., 2015; García-Gonzalo et al., 2015). Recent studies using immortalized Inpp5eΔ/Δ MEFs suggests INPP5E regulates the ciliary level of the Hh suppressor GPR161 via TULP3, that in turn promotes GLI3R formation and reduced Hh signaling (Mukhopadhyay et al., 2013; Garcia-Gonzalo et al., 2015). Here, we reveal Inpp5e ablation suppresses Hh signaling associated with aberrant Hh-dependent embryonic patterning in vivo including reduced “high level” and expansion of “intermediate level” Hh signaling. We investigated the mechanisms leading to the loss of high-level Hh signaling with Inpp5e deletion, and our genetic studies provide evidence that INPP5E contributes to Hh signaling regulation during embryonic development and that perturbation of...
this pathway underlies several disease phenotypes associated with Inpp5e deletion. Anophthalmia is typically associated with repressed Hh signaling (Bakrania et al., 2010) and was observed in all Inpp5e<sup>−/−</sup> embryos at E15.5. Rescue of the anophthalmia in Inpp5e<sup>−/−</sup>;CMVCre;SmoM2 embryos suggests SOMOM2 expression in the context of Inpp5e deletion reequilibrates Hh signaling in vivo (Bakrania et al., 2010; Slavotinek, 2011). Supporting this, we observed normalization of Hh target gene expression in Inpp5e<sup>−/−</sup>;CMVCre;SmoM2 embryos. Notably, GPR161 cilia levels were increased in rescue Inpp5e<sup>−/−</sup>;CMVCre;SmoM2 pMEFs, suggesting the partial phenotypic rescue may be a consequence of increased GPR161 at cilia, and thus, the deregulation of SMO and GPR161 at cilia may additively impact the in vivo phenotypes of Inpp5e<sup>−/−</sup> mice.

Inpp5e<sup>−/−</sup> embryos exhibit normal cilia number but reduced Hh signaling, suggesting a defect in the coordination of Hh signal transduction. We demonstrate INPP5E is essential for maximal SMO accumulation at cilia after the Hh pathway; however, some Inpp5e<sup>−/−</sup> null cells showed low-level SMO cilia accumulation, suggesting some modes of SMO trafficking are intact. We propose a defect in cilia receptor retention may underlie the reduced cilia localization of SMO in Inpp5e<sup>−/−</sup> null cells. Consequent with this, cilia entry is unperturbed with Inpp5e deletion, but the cilia accumulation of receptors, including SMO and polycystin II, is reduced specifically after Hh pathway activation. FRAP analysis revealed an increase in the cilia exchange of SMO in Inpp5e<sup>−/−</sup> pMEFs, collectively suggesting the barrier properties of the TZ that normally act to limit SMO diffusion out of the cilium are impaired with Inpp5e deletion. Importantly, we show the 5-phosphatase activity of INPP5E, which regulates the levels of PI(4,5)P<sub>2</sub> and PI(3,4,5)P<sub>3</sub> at the TZ, is essential for SMO accumulation at cilia as well as Hh signaling and that expression of constitutively active SMOM2 partially rescues Inpp5e<sup>−/−</sup> null embryonic defects. Therefore, we propose the reduced cilia retention of SMO is at least in part responsible for some Inpp5e<sup>−/−</sup> phenotypes.

The TZ plays an essential role in the cilia accumulation of receptors; however, the molecular mechanisms underlying TZ function are still emerging. Several studies suggest the assembly of protein scaffolds at the TZ as a proteinaceous gate that contributes to TZ barrier function, and other studies propose septins play a role (Hu et al., 2010; Chih et al., 2011; Garcia-Gonzalo et al., 2011; Huang et al., 2011; Williams et al., 2011; Roberson et al., 2015). TMEM231 and B9D1 cooperatively localize to the TZ, and SEPT2 localizes to the TZ scaffold components, which in turn maintain SMO at cilia. The protein scaffold components MKS1, TCTN1, TMEM231, and B9D1 cooperatively localize to the TZ, and SEPT2 localizes TMEM231 and B9D1 to the TZ, and all are essential for TZ function (Chih et al., 2011; Garcia-Gonzalo et al., 2011; Williams et al., 2011; Roberson et al., 2015). TMEM231 and TMEM17 may anchor B9 complex components, including TCTN1 and B9D1, to the TZ membrane and in this way act as a “protein fence” to limit diffusion of ciliary membrane proteins. Here, we show that Hh-stimulated, but not steady-state, Inpp5e<sup>−/−</sup> pMEFs exhibit reduced TZ localization of MKS1, TCTN1, TMEM231, and B9D1, which is associated with increased PI(4,5)P<sub>2</sub> and PI(3,4,5)P<sub>3</sub> signals at the TZ. Significantly, expression of wild-type, but not phosphatase-dead, INPP5E restored the localization of TZ scaffold components in Hh-stimulated Inpp5e<sup>−/−</sup> pMEFs, revealing INPP5E regulation of PI(4,5)P<sub>2</sub> and/or PI(3,4,5)P<sub>3</sub> signaling at the TZ contributes to TZ function. PI<sub>S</sub> are integral for the identity of cellular and organelle membranes as well as membrane microdomains, and they serve as compartment-specific recognition signals for the recruitment of transmembrane or cytosolic proteins (Carlton and Cullen, 2005). Many TZ components, including MKS1, TCTN1, and TMEM231, contain transmembrane domains and/or multiple modules such as coiled-coil, B9, and C2 domains that mediate their TZ localization and facilitate scaffold assembly (Arts et al., 2007; Delous et al., 2007; Williams et al., 2011; Jensen et al., 2015). Significantly, these protein modules are predicted to mediate PI binding or enhance protein–membrane or protein–protein avidity via coincidence detection (Nalefski and Falke, 1996; Lemmon, 2008; Jensen et al., 2015). Similarly, septins bind various PI<sub>S</sub> and septic oligomerization that is integral to its TZ barrier function is regulated by PIs in a concentration-dependent manner (Casamayor and Snyder, 2003; Badrane et al., 2012; Bridges et al., 2014).

As shown here, the level of both PI(4,5)P<sub>2</sub> and PI(3,4,5)P<sub>3</sub> changes at the TZ after Hh pathway activation, raising the possibility that the PI(4,5)P<sub>2</sub>/PI(3,4,5)P<sub>3</sub> signal ratio itself plays a role in TZ function. Indeed PI(4,5)P<sub>2</sub>/PI(3,4,5)P<sub>3</sub> composition and ratio contribute to membrane identity and the targeting of proteins to other membrane subdomains (Heo et al., 2006). Interestingly, inhibition of PI3K in Hh-stimulated Inpp5e<sup>−/−</sup> null pMEFs partially restored the TZ localization of TCTN1, TMEM231, and B9D1, suggesting a specific role for PI(3,4,5)P<sub>3</sub>; however, further studies are required to determine the individual and/or cooperative roles of PI(4,5)P<sub>2</sub> and PI(3,4,5)P<sub>3</sub> in TZ molecular organization and function.

We showed that PI(4,5)P<sub>2</sub> and PI(3,4,5)P<sub>3</sub> signals are both modulated at the TZ by Hh signaling, whereby PI(4,5)P<sub>2</sub> signals decrease and PI(3,4,5)P<sub>3</sub> signals increase after Hh pathway activation. The simplest interpretation of this profile is that PI(4,5)P<sub>2</sub> is constitutively localized to the TZ in quiescent cells and with induction of Hh signaling PI3K is activated, allowing for PI(4,5)P<sub>2</sub> phosphorylation to generate PI(3,4,5)P<sub>3</sub>, resulting in a concomitant decrease in PI(4,5)P<sub>2</sub> and increase in PI(3,4,5)P<sub>3</sub> signals. Our model proposes Inpp5e deletion amplifies Hh-stimulated changes in PI(4,5)P<sub>2</sub> and PI(3,4,5)P<sub>3</sub> signals at the TZ, resulting in misorganization and dysfunction of the TZ and reduced levels of SMO at cilia with suppression of GLI2 cilia tip localization, resulting in reduced Hh signaling (Fig. 8). Others have recently reported INPP5E regulates the cilia enrichment of the Hh suppressor GPR161, which is recruited to cilia via TULP3 that binds PI(4,5)P<sub>2</sub> (Mukhopadhyay et al., 2010; Chávez et al., 2015; Garcia-Gonzalo et al., 2015). In this way, increased axoneme PI(4,5)P<sub>2</sub> signals with Inpp5e deletion may enhance TULP3-mediated GPR161 cilia recruitment, which acts to suppress Hh signaling by promoting GLI3R formation (Mukhopadhyay et al., 2013; Garcia-Gonzalo et al., 2015). Here, we showed GPR161 is increased at cilia in a subpopulation of quiescent Inpp5e<sup>−/−</sup> pMEFs and that after Hh pathway activation, GPR161 is partially removed from cilia.
but levels remain increased compared with wild type, consistent with other studies (Garcia-Gonzalo et al., 2015). No known Hh pathway agonists directly regulate GPR161 signaling. However, active SMO at cilia is essential for the removal of GPR161 from cilia after SAG stimulation by promoting GPR161 binding to β-arrestin and thereby GPR161 endocytosis (Pal et al., 2016). Therefore, the regulation of GPR161 by INPP5E may be integrated and downstream of the INPP5E–SMO signaling axis identified here, allowing for the fine-tuning of Hh signaling.

In summary, we identify INPP5E regulation of PI(4,5)P2/PI(3,4,5)P3 at the TZ as a novel mechanism that contributes to TZ function. INPP5E regulation of SMO accumulation at cilia is essential for normal Hh signaling and therefore embryonic development. Collectively, our studies reveal a cooperative signaling network among INPP5E, PI3K, and Hh signaling at the TZ.

**Materials and methods**

**Antibodies, constructs, and reagents**

Antibodies and organelle stains used were ac-tubulin (Sigma-Aldrich), ARL13B (Neuromab), B9D1 (Novus Biologicals), DAPI (Sigma-Aldrich), FLAG (Sigma-Aldrich), GAPDH (Ambion), GLI1 (Santa Cruz Biotechnology, Inc.), GLI2 (Santa Cruz Biotechnology, Inc.), GPR161 (Proteintech), HA (Covance), HA (Roche), Islet1 (Developmental Studies Hybridoma Bank [DSHB]), Map2 (Cell Signaling Technology), MKS1 (Proteintech), Nkx2.2 (DSHB), Pax6 (DSHB), Pericentrin (Covance), polycystin 2 (Santa Cruz Biotechnology, Inc.), PI(4,5)P2 (Echelon), PI(3,4,5)P3 (Echelon), SEPT2 (Sigma-Aldrich), Shh (DSHB), SMO (R. Rohatgi, Stanford University, Stanford, CA; Rohatgi et al., 2007), SMO (Lifespan Biosciences), TCTN1 (Protein-tech), TMEM231 (Novus Biologicals), and γ-tubulin (Thermo Fisher Scientific). Alexa Fluor® or HRP-conjugated secondary antibodies were from Thermo Fisher Scientific and GE Healthcare, respectively. All other reagents were from Sigma-Aldrich unless otherwise specified. The Islet1, Nkx2.2, Pax6, and Shh monoclonal antibodies developed by T.M. Jessell and S. Brenner-Morton (Columbia University, New York, NY) were obtained from the DSHB developed under the auspices of the National Institute of Child Health and Human Development and maintained by the Department of Biology at The University of Iowa (Iowa City, IA), pcGN (vector), pcGN-INPP5E (HA-INPP5E), and pcGN-INPP5E(D480N; HA-INPP5EΔNLS) were described previously (Kong et al., 2000, 2006). Ves-INPP5E was described previously (Plotnikova et al., 2015). GFP-PH(Btk) and GFP(PLC) were gifts from T. Balla (National Institutes of Health, Bethesda, MD). HTR6-Tango was a gift from B. Roth (University of North Carolina, Chapel Hill, NC; plasmid 66414; Addgene; Krooe et al., 2015), mEmerald-MKS1-N-18 was a gift from M. Davidson (Florida State University, Tallahassee, FL; plasmid 54183; Addgene), pCS2+ YFP-SMO was a gift from R. Rohatgi (plasmid 41086; Addgene; Dorn et al., 2012), CEACAM-1-GFP was purchased from OriGene.

**Mouse strains**

All animal work conformed to ethical standards for animal handling and was approved by MARP2 Monash University animal ethics committee. A targeting construct was generated with loxp sites inserted between exons 1 and 2 and exons 6 and 7 and a FRT site–flanked neomycin cassette inserted between exons 6 and 7 of the mouse Inpp5e gene. Homologous recombination using the targeted Inpp5e construct was performed in embryonic stem cells and neomycin-resistant clones selected. Recombinant clones were used to generate C57BL/6 chimeric mice. Progeny of the chimeric mice containing the targeted Inpp5e allele were crossed with CMVCre, which were then backcrossed to C57BL/6 mice to remove the CMVCre transgene. Heterozygous Inpp5e+/−/Cre+ matings were used to generate Inpp5e−/−/Cre− embryos.

**Materials and methods**

**Antibodies, constructs, and reagents**

Antibodies and organelle stains used were ac-tubulin (Sigma-Aldrich), ARL13B (Neuromab), B9D1 (Novus Biologicals), DAPI (Sigma-Aldrich), FLAG (Sigma-Aldrich), GAPDH (Ambion), GLI1 (Santa Cruz Biotechnology, Inc.), GLI2 (Santa Cruz Biotechnology, Inc.), GPR161 (Proteintech), HA (Covance), HA (Roche), Islet1 (Developmental Studies Hybridoma Bank [DSHB]), Map2 (Cell Signaling Technology), MKS1 (Proteintech), Nkx2.2 (DSHB), Pax6 (DSHB), Pericentrin (Covance), polycystin 2 (Santa Cruz Biotechnology, Inc.), PI(4,5)P2 (Echelon), PI(3,4,5)P3 (Echelon), SEPT2 (Sigma-Aldrich), Shh (DSHB), SMO (R. Rohatgi, Stanford University, Stanford, CA; Rohatgi et al., 2007), SMO (Lifespan Biosciences), TCTN1 (Protein-tech), TMEM231 (Novus Biologicals), and γ-tubulin (Thermo Fisher Scientific). Alexa Fluor® or HRP-conjugated secondary antibodies were from Thermo Fisher Scientific and GE Healthcare, respectively. All other reagents were from Sigma-Aldrich unless otherwise specified. The Islet1, Nkx2.2, Pax6, and Shh monoclonal antibodies developed by T.M. Jessell and S. Brenner-Morton (Columbia University, New York, NY) were obtained from the DSHB developed under the auspices of the National Institute of Child Health and Human Development and maintained by the Department of Biology at The University of Iowa (Iowa City, IA), pcGN (vector), pcGN-INPP5E (HA-INPP5E), and pcGN-INPP5E(D480N; HA-INPP5EΔNLS) were described previously (Kong et al., 2000, 2006). Ves-INPP5E was described previously (Plotnikova et al., 2015). GFP-PH(Btk) and GFP(PLC) were gifts from T. Balla (National Institutes of Health, Bethesda, MD). HTR6-Tango was a gift from B. Roth (University of North Carolina, Chapel Hill, NC; plasmid 66414; Addgene; Krooe et al., 2015), mEmerald-MKS1-N-18 was a gift from M. Davidson (Florida State University, Tallahassee, FL; plasmid 54183; Addgene), pCS2+ YFP-SMO was a gift from R. Rohatgi (plasmid 41086; Addgene; Dorn et al., 2012), CEACAM-1-GFP was purchased from OriGene.

**Mouse strains**

All animal work conformed to ethical standards for animal handling and was approved by MARP2 Monash University animal ethics committee. A targeting construct was generated with loxp sites inserted between exons 1 and 2 and exons 6 and 7 and a FRT site–flanked neomycin cassette inserted between exons 6 and 7 of the mouse Inpp5e gene. Homologous recombination using the targeted Inpp5e construct was performed in embryonic stem cells and neomycin-resistant clones selected. Recombinant clones were used to generate C57BL/6 chimeric mice. Progeny of the chimeric mice containing the targeted Inpp5e allele were crossed with Flp recombinase transgenic mice to generate Inpp5e+/−;F1p mice (Hakim et al., 2016). Inpp5e+/−;F1p mice were backcrossed with Inpp5e−/−; C57BL/6 mice to remove the recombinase. Inpp5e−/− mice were then crossed with CMVCre transgenic mice to generate Inpp5e−/−; CMVCre mice, which were then backcrossed with Inpp5e−/−; C57BL/6 mice to remove the CMVCre transgene. Heterozygous Inpp5e−/+ matings were used to generate Inpp5e−/− embryos.
Australia). To generate Inpp5e<sup>−/−</sup>;CMVCre;SmoM2<sup>−/−</sup> mice, Inpp5e<sup>−/−</sup> mice were crossed with mice expressing constitutively active SmoM2 to generate Inpp5e<sup>−/−</sup>;SmoM2<sup>−/−</sup> mice. Inpp5e<sup>−/−</sup> mice were then crossed with mice expressing CMVCre recombinase to generate Inpp5e<sup>−/−</sup>;CMVCre mice that were then crossed with Inpp5e<sup>−/−</sup>;SmoM2<sup>−/−</sup> mice to generate Inpp5e<sup>−/−</sup>;CMVCre;SmoM2<sup>−/−</sup> mice.

**Cell culture and transient transfections**

hTERT-RPE1 (RPE) cells were cultured in DMEM-F12 with 10% FCS and 0.01 mg/ml hygromycin and were transfected using Lipofectamine 2000 per the manufacturer's instructions. For isolation of pMEFs, E12.5 embryos were decapitated and eviscerated and the embryonic tissue minced and incubated in 0.25% trypsin-EDTA using a standard protocol. pMEFs were transfected via nucleofection using the Lonza MEF2 kit with a transfection efficiency of ~80%.

**Immunofluorescence and image analysis**

For microscopy, RPE cells or pMEFs were grown on coverslips and serum starved for 24 or 48 h, respectively, to induce cilia and, where indicated, treated with 100 nM SAG for 12–24 h. For PI3K inhibition and PI(3,4,5)P<sub>3</sub> antibody validation, cells were treated with 10 μM LY294002 at 37°C for 1 h before fixation. For PI(4,5)P<sub>2</sub> antibody validation, cells were treated after fixation with 10 μM neomycin for 30 min. For detection of PI(4,5)P<sub>2</sub> at cilia, the “Golgi” staining protocol was used as described previously (Hammond et al., 2009). For detection of PI(3,4,5)P<sub>3</sub> in perineural cilia, E10.5 mouse embryos were embedded in paraffin and 8-μm sections processed via standard protocols. To examine neural tube and perineural cilia, E10.5 mouse embryos were embedded in paraffin and 8-μm sections processed via standard immunohistochemical procedures. For whole-mount in situ hybridization, limb buds were hybridized with riboprobes for Ptc1, GLI1, and HoxD13 as previously described (Fowles et al., 2003).

**Quantitative RT-PCR**

To quantitate Ptc1 mRNA levels in pMEFs, RNA was extracted using the RNaseasy mini kit (QIAGEN) and cDNA synthesis performed using the AffinityScript QPCR cDNA Synthesis kit (Agilent Technologies) according to the manufacturer’s instructions. Quantitative RT-PCR was performed on the 7900H Fast Real-Time PCR system (Applied Biosystems) using TaqMan 2× PCR MasterMix and Ptc1 and Acb1 TaqMan probes (Applied Biosystems). Each sample was analyzed in triplicate and normalized to Acb1 expression.

To quantify Gli1 mRNA levels in pMEFs and Ptc1 levels in E10.5 embryos, RNA was isolated using the Isolate RNA mini kit (Bioline) and cDNA synthesis performed using the AffinityScript QPCR cDNA Synthesis kit (Agilent Technologies) according to manufacturer’s instructions. Quantitative RT-PCR was performed on a Rotor-Gene 3000-qRT-PCR machine using Brilliant II SYBR Green qPCR Master Mix (Agilent Technologies) and Gli1, Ptc1, and Gapdh primers (QIAGEN). Each sample was analyzed in triplicate and normalized to Gapdh expression.

**Image processing and analysis**

Image analysis and processing were performed using FIJI software. To determine the MFI and area of a “candidate” at the TZ, an ROI outlining the ac-tubulin axoneme was transposed onto the candidate channel. The signal proximal to the base of the axoneme was selected and the “mean gray value” and “area” measured and expressed as arbitrary units. To determine the GLI2 MFI specifically at the cilium tip, the ROI encompassing the ac-tubulin axoneme was transposed onto the GLI2 channel. The GLI2 signal detected distal to the ac-tubulin signal (representing the extreme cilium tip) was selected as the ROI and the mean grey value measured and expressed as arbitrary units. To determine the candidate/ac-tubulin ratio, the MFI in the ROI was measured...
for the candidate and the corresponding ac-tubulin signal and the ratio calculated and expressed as arbitrary units.

**Statistical analysis**

Statistical analysis was performed on data from independent experiments. Graphs were generated using GraphPad Software Prism6 and represent mean ± SEM. Statistical significance was evaluated using unpaired two-tailed Student’s t test and confirmed using the Mann–Whitney U test for all experiments, with the exception of mice genotype frequency analysis. A χ² test was used to compare the observed genotypic frequency versus Mendelian ratios (Fig. 2 mouse studies and Table S1). Differences were considered significant when P < 0.05.

**Online supplemental material**

Fig. S1 shows the phenotype of Inpp5e−/− embryos, including developmental abnormalities, aberrant Hh-dependent tissue patterning, reduced Hh signaling, and normal cilia number. Fig. S2 shows the partial rescue of Inpp5e−/− phenotypes via expression of constitutively active SMOM2. Fig. S3 shows the cilia localization of wild-type HA-INPP5E and 5-phosphatase-dead HA-INPP5E/ΔNPP, as well as the reduced cilia accumulation of FLAG-HTR6 and the cilia exclusion of GFP–CEACAM-1 in Hh-stimulated Inpp5e−/− pMEFs. Fig. S4 shows Pl(4,5)P₂ and Pl(3,4,5)P₃ localize to the cilia TZ and that Pl(4,5)P₂ and Pl(3,4,5)P₃ are regulated at cilia by Hh signaling and INPP5E. Fig. S5 shows the colocalization of endogenous TZ markers with a recombinant TZ protein (Emerald-MKS1) to confirm antibody specificity. In addition, the mislocalization of SEPT2 in Hh-stimulated Inpp5e−/− pMEFs is shown. Table S1 lists the penetrance of phenotypes in E15.5 Inpp5e−/−;CMVCre, Inpp5e+/−;CMVCre;SmoM2 and Inpp5e−/−;CMVCre;SmoM2 embryos.

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**References**


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