RESEARCH ARTICLE

Identification of disease-relevant modulators of the SHH pathway in the developing brain

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ABSTRACT

Pathogenic gene variants in humans that affect the sonic hedgehog (SHH) pathway lead to severe brain malformations with variable penetrance due to unknown modifier genes. To identify such modifiers, we established novel congenic mouse models. LRP2-deficient C57BL/6N mice suffer from heart outflow tract defects and holoprosencephaly caused by impaired SHH activity. These defects are fully rescued on a FVB/N background, indicating a strong influence of modifier genes. Applying comparative transcriptomics, we identified Ptg1 and Ulk4 as candidate modifiers upregulated in the rescue strain. Functional analyses showed that ULK4 and PTTG1, both microtubule-associated proteins, are positive regulators of SHH signaling, rendering the pathway more resilient to disturbances. In addition, we characterized ULK4 and PTTG1 as previously unidentified components of primary cilia in the neuroepithelium. The identification of genes that powerfully modulate the penetrance of genetic disturbances affecting the brain and heart is likely relevant to understanding the variability in human congenital disorders.

KEY WORDS: SHH, Primary cillum, Forebrain development, Holoprosencephaly, Heart, Modifier genes

INTRODUCTION

Holoprosencephaly (HPE) is the most common structural defect of human forebrain development. HPE is characterized by impaired separation of the cerebral hemispheres and is typically accompanied by craniofacial malformations (Geng and Oliver, 2009; Hong and Krauss, 2018; Krauss, 2007; Ming and Muenke, 2002; Muenke and Beachy, 2000). During embryonic forebrain development, HPE can be induced by exposure to environmental toxins (Krauss and Hong, 2016; Weiss et al., 2018) or can be due to genetic predisposition with mono- or polygenic contributions (Krauss and Hong, 2016; Roessler and Muenke, 2010). Single mutations causing HPE have been identified (Hayhurst and McConnell, 2003; Roessler and Muenke, 2010; Roessler et al., 1996) and, among those, mutations in human sonic hedgehog (SHH) and its downstream effector genes account for at least 5% of autosomal dominant nonsyndromic HPE cases (Wallis and Muenke, 1999, 2000; Wallis et al., 1999). However, even within pedigrees carrying the same SHH mutation, HPE phenotypes vary among relatives and can range from alobar HPE, to facial abnormalities typical of HPE, to asymptomatic appearance of the carrier. Such intrafamilial variability of HPE phenotypes could be due to both environmental and genetic factors (Heussler et al., 2002; Hong and Krauss, 2018; Ming and Muenke, 2002; Muenke and Beachy, 2000; Muenke and Cohen, 2000; Roessler et al., 1996).

Studies on mouse models for HPE suggest that loss-of-function mutations in genes, relevant for forebrain development, result in brain and craniofacial anomalies that recapitulate HPE phenotypes in patients carrying mutations in the same genes (Geng and Oliver, 2009; Hayhurst and McConnell, 2003; Heyne et al., 2016; Hong and Krauss, 2018). Mice deficient for SHH develop forebrain defects resembling those of human HPE cases (Chiang et al., 1996). Intriguingly, the variability of HPE phenotypes found in humans is also reflected in gene-targeted mouse models, as phenotype penetrance often depends on the mouse strain (Anderson et al., 2002; Cole and Krauss, 2003; Geng and Oliver, 2009; Geng et al., 2008; Hong and Krauss, 2018; Petryk et al., 2004; Schachter and Krauss, 2008).

LRP2 (low-density lipoprotein receptor-related protein 2) is a component of the SHH signaling machinery in the primary cillum (Christ et al., 2012) and has been implicated in the etiology of HPE in mice and humans (Kim et al., 2019; Rosenfeld et al., 2010; Spoelgen et al., 2005; Willnow et al., 1996). Humans with autosomal recessive LRP2 gene defects suffer from Donnai-Barrow syndrome, with clinical characteristics including heart anomalies (Pober et al., 2009), craniofacial anomalies, forebrain defects (Kantarcı et al., 2007; Ozdemir et al., 2019) and microforms of HPE (Rosenfeld et al., 2010). In addition, severe forms of HPE in families presenting with oligogenic events involving LRP2 have been identified (Kim et al., 2019), and inter- and intrafamilial phenotypic variability, which is a characteristic feature of HPE (Dubourg et al., 2018; Solomon et al., 2010), is observed in Donnai-Barrow syndrome (Khalifa et al., 2015; Longoni et al., 2008; Pober et al., 2009).

Here, we have analyzed two congenic mouse models of Lrp2 that reflect the phenotypic variability of HPE in humans with LRP2 mutations. Comparison of these Lrp2−/− strains allowed us to...
identify candidate modifier genes that affect SHH signaling in the primary cilium.

RESULTS
Genetic background determines penetrance of craniofacial, brain and heart defects in Lrp2−/− mutant mice

Lrp2−/− mice on a C57BL/6N background at embryonic stage (E) 18.5 displayed severe HPE-associated craniofacial dysgenesis (Fig. 1A). In particular, they showed cleft lip (Fig. 1A, arrow) and incompletely developed or even bilaterally or unilaterally absent eyes (Fig. 1A, asterisk). Further characteristic features in these mutants were a shortened skull and an open anterior suture (Fig. 1A, arrowhead). In contrast, Lrp2−/− congenic mice with a FVB/N background showed 100% penetrant rescue of all the above described craniofacial defects (Fig. 1A), except for a mild suture phenotype (Fig. 1A, arrowhead). HPE in Lrp2−/− C57BL/6N embryos, resulting from impaired ventral midline specification, was fully penetrant and characterized by a fusion of the cortical hemispheres (Fig. 1B, arrowheads) with a single lateral ventricle (Fig. 1B, arrow) and absent olfactory bulbs (Fig. 1B, asterisks). In contrast, HPE was fully rescued in all Lrp2−/− FVB/N mutants, which presented with correctly separated cortical hemispheres, olfactory bulbs and clearly defined lateral ventricles. Corpus callosum and the anterior commissure were present in the LRP2-deficient FVB/N mice (Fig. 1B).

LRP2-deficient C57BL/6N mice also suffer from cardiovascular anomalies, including a common arterial trunk (CAT) (Baardman et al., 2016; Christ et al., 2020; Li et al., 2015). Interestingly, there is comorbidity of HPE and congenital heart disease in individuals with variants of known HPE-related genes (Tekendo-Ngongang et al., 2020).

Lrp2−/− embryos with a CAT display a single common coronary outflow artery vessel instead of a pulmonary artery and an ascending aorta. Less frequently, Lrp2−/− embryos displayed a double outlet right ventricle (DORV), in which the pulmonary artery and ascending aorta both connect to the right ventricle. We found that 15 out of 16 Lrp2−/− mutants on a C57BL/6N background displayed CAT (Fig. S1C,D,F,F′ and Table S1), and one remaining mutant embryo displayed a DORV (Table S1). The CAT phenotype was also visualized by colored liquid plastic injection (Fig. S1D, arrowhead), which indicated a CAT and a ventricular septum defect. We never observed a CAT in Lrp2−/− FVB/N mutants; 85% of Lrp2−/− FVB/N embryos showed no

Fig. 1. Rescue of craniofacial malformations, HPE and heart defects in Lrp2−/− FVB/N mice. (A) Lrp2+/− C57BL/6N E18.5 embryos (n=30) with craniofacial defects: underdeveloped or missing eyes (asterisk), cleft lip (arrow) and hypertelorism. Dorsal view of the head revealed a shortened and widened skull with impaired suture formation and an open fontanelle (arrowhead). Lrp2+/− and Lrp2+/− littermates served as controls (n=66). Lrp2−/− FVB/N mutants (n=28) displayed normal skull formation, normal eyes and normal midface structures, comparable with controls (n=94). Lrp2−/− FVB/N embryos had a mild incomplete anterior fontanelle closure (arrowhead). Pigmentation of the retina is missing due to albinism in FVB/N. Scale bars: 1 mm. (B) Brains from E18.5 control and Lrp2−/− embryos in dorsal (d) and ventral (v) view, and Nissl-stained coronal sections. Lrp2−/− C57BL/6N brain with alobar HPE (arrowheads) (Lrp2−/− n=4; controls n=6). Olfactory bulbs were absent in mutants (asterisks). All sectioned Lrp2−/− C57BL/6N brains (n=13) showed alobar HPE with a single lateral ventricle (arrow) compared with controls (n=6). Corpus callosum (CC) and anterior commissure (AC) were missing in Lrp2−/− C57BL/6N mice. Lrp2−/− FVB/N embryos showed normal forebrain separation (Lrp2−/− n=5; controls n=3). Olfactory bulbs were present in Lrp2−/− FVB/N brains. All Lrp2−/− FVB/N sectioned brains (n=6) displayed normally separated ventricles, and a normal CC and AC comparable with controls (n=4). Scale bars: 1 mm.
apparent heart anomalies and a normal ventricular septum compared with controls (Fig. S1L, I, L’ and Table S1). Two out of 13 Lrp2−/− FVB/N mice showed a DORV (Table S1), which has been described in mutants on C57BL/6N strain at a similar frequency (Baardman et al., 2016). We conclude that the FVB/N background fully rescues the CAT phenotype, whereas the etiology of a DORV is not influenced by the strain background, suggesting the likelihood of divergent pathogenic mechanisms for a CAT and a DORV in Lrp2−/− mutants.

**Rescue of SHH activity in neuroepithelial stem cells**

In the neuroepithelium, LRP2 is located at the base of the primary cilium, where it teams up with patched 1 to mediate endocytic uptake and recycling of SHH (Christ et al., 2012). Consistent with our previous findings, Lrp2−/− C57BL/6N embryos showed normal SHH pattern in the prechordal plate, but little to no SHH in the overlying neuroepithelial stem cells compared with control embryos during the critical stages of forebrain specification at the 8-somite stage (Fig. 2B, 8 s). SHH protein was detected later in the neuroepithelium at the 11-somite stage, which is too late for proper ventral midline specification (Fig. 2B, 11 s). Intriguingly, all Lrp2−/− FVB/N mutants showed completely normal SHH localization throughout all developmental stages (Fig. 2B). To exclude a later onset of defects, we analyzed later embryonic stages. A fully penetrant hallmark of the Lrp2−/− C57BL/6N embryos at E10.5 is the lack of Shh RNA and protein in the telencephalon (Christ et al., 2012; Spoelgen et al., 2005) (Fig. 2C, arrowhead; Fig. S2A, arrowhead) and in the ventral midline of the diencephalon (Fig. S2B, arrowhead). Lrp2−/− FVB/N mutants showed normal Shh RNA expression and SHH protein localization in the ventral telencephalon and diencephalon (Fig. 2C, arrowhead; Fig. S2A,B).

Normal Nkx2.1 expression pattern comparable with controls (Fig. S2G,H) confirmed proper activation of SHH downstream targets and intact ventral forebrain patterning in Lrp2−/− FVB/N mice (Fig. S2L,J), in contrast to mutants on a C57BL/6N background, which lacked Nkx2.1 expression in the ventral forebrain (Fig. S2E,F) compared with controls (Fig. S2C,D). Thus, defects in the SHH pathway caused by loss of LRP2 were fully rescued on a FVB/N background. Consequently, the severe telencephalic vesicle separation defects in Lrp2−/− C57BL/6N mutants (Fig. S2K) did not manifest in mutants on a FVB/N background displaying properly separated forebrain hemispheres at E12.5 (Fig. S2K). Of note, Lrp2−/− mutants on both C57BL/6N and FVB/N backgrounds showed a more dilated and widened dorsal forebrain compared with the controls as shown in frontal views on whole embryos (Fig. S2K, asterisks) and on coronal sections (Fig. S2E,F,I,J, asterisks). The dilation of the dorsal neural tube is unrelated to impaired SHH function in the ventral neural tube and conserved between mouse strains and Xenopus with LRP2 loss of function (Kowalczyk et al., 2021).

**Dominant effect of the FVB/N-specific transcriptome**

To test a possible dominant effect of FVB/N-specific candidate modifier genes, we collected Lrp2+/− and Lrp2−/− first-generation hybrids from Lrp2+/− C57BL/6N×Lrp2−/− FVB/N parents (hereafter referred to as F1 embryos). Detailed phenotypic analyses of Lrp2−/− F1 embryos indeed revealed a full rescue of brain and craniofacial defects. They suffered from neither HPE nor
cleft lip (Fig. 3A and Fig. S3A). Analyzing the heart by stereomicroscopy, we never observed a common outflow tract in *Lrp2*<sup>−/−</sup> F1 embryos (0/27) at E18.5. Applying polymeric dye injection and histology revealed that 56% of the *Lrp2*<sup>−/−</sup> F1 embryos, we detected a DORV (Fig. S3E,F,G,G′ and Table S1). In 44% of the *Lrp2*<sup>−/−</sup> F1 embryos, we concluded that FVB/N allele-specific expression of yet unidentified genes has a dominant rescue effect. We hypothesize that such factors predispose neuroepithelial stem cells in FVB/N and F1 mice to maintain sufficient SHH signaling, despite the loss of LRP2, and thereby prevent HPE.

To identify factors conveying disease resistance, we next analyzed the transcriptome of *Lrp2*<sup>+/+</sup> and *Lrp2*<sup>−/−</sup> embryonic heads from C57BL/6N, FVB/N and F1 backgrounds (Fig. 3C). Performing DESeq2 differential expression analyses on all transcriptomes (Fig. 3D heatmap and Table S2), we detected 2170 differentially expressed genes (DEGs) between *Lrp2*<sup>−/−</sup> and *Lrp2*<sup>+/+</sup> embryos on a C57BL/6N background, only 367 DEGs for the FVB/N background and a similarly low number (241 DEGs) for the F1 background (Fig. 3E). Expression levels of HPE- and SHH pathway-related transcription factors such as *Vax1*, *Six6* and *Six3* were significantly decreased in *Lrp2*<sup>−/−</sup> C57BL/6N mutants compared with controls, but were unchanged in *Lrp2*<sup>−/−</sup> FVB/N mutants compared with FVB/N controls (Fig. 3F). In addition, the *Lrp2*<sup>−/−</sup> F1 mutants showed normal expression levels for *Vax1*, *Six6* and *Six3*, comparable with the *Lrp2*<sup>−/−</sup> controls (Fig. 3F), reflecting the rescue phenotype and suggesting a dominant effect of the FVB/N transcriptome.

**Identification of strain-specific candidate modifier genes involved in ciliogenesis and SHH pathway regulation**

To identify strain-specific candidate modifier genes and pathways underlying HPE susceptibility in C57BL/6N, and HPE rescue in FVB/N and F1, we used the transcriptome data set described above (Table S2). However, in this approach we focused on the strain-specific transcriptome differences. Thus, we compared gene expression between mutant *Lrp2*<sup>−/−</sup> samples on C57BL/6N, FVB/N and F1 backgrounds (Fig. 4A-E) and, most importantly, also between wild-type (*Lrp2*<sup>+/+</sup>) samples comparing C57BL/6N, FVB/N and F1 backgrounds (Fig. 4A-E). We found that wild-type (*Lrp2*<sup>+/+</sup>) samples on a C57BL/6N background (2170 DEGs), FVB/N background (367 DEGs) and F1 background (241 DEGs) showed significant differences in gene expression compared with mutant samples. Specifically, expression levels of HPE- and SHH pathway-related transcription factors such as *Vax1*, *Six6* and *Six3* were significantly decreased in *Lrp2*<sup>−/−</sup> C57BL/6N mutants compared with controls, but were unchanged in *Lrp2*<sup>−/−</sup> FVB/N mutants compared with FVB/N controls (Fig. 3F). In addition, the *Lrp2*<sup>−/−</sup> F1 mutants showed normal expression levels for *Vax1*, *Six6* and *Six3*, comparable with the *Lrp2*<sup>−/−</sup> controls (Fig. 3F), reflecting the rescue phenotype and suggesting a dominant effect of the FVB/N transcriptome.
FVB/N and F1 backgrounds (Fig. 4F-J). Venn diagrams show the number of DEGs in Lrp2−/− embryos comparing the different backgrounds (Fig. 4C) and in wild-type Lrp2+/+ embryos accordingly (Fig. 4H). Given the dominant effect of the FVB/N background, we focused on DEGs that were identified in both comparisons: C57BL/6N versus FVB/N and C57BL/6N versus F1 (Fig. 4D,I and Table S2). Importantly, out of the 1044 DEGs from the analysis comparing Lrp2−/− mutant samples, 426 DEGs were also identified in the wild-type Lrp2+/+ comparisons (Table S2). Thus, these DEGs, which are common between mutant and wild-type comparisons of different strains, were not a consequence of LRP2 loss of function but were truly strain specific, and therefore met our criteria for strain-specific modifier candidates that can modulate signaling pathways in the wild-type situation.

As there was a clear effect on the SHH pathway in the ventral forebrain, we next filtered those DEGs with a known function in the SHH pathway, cilia or ciliopathies from our strain-specific datasets. Hedgehog and cilia/ciliopathy relevant genes were selected according to published data (Breslow et al., 2018; Pusapati et al., 2018), which include the Syscilia gold standard, and their expression was visualized by volcano plots (Fig. 4E,J and Table S3). We determined that among cilia- and SHH-related genes, 12 were downregulated and 10 were upregulated in Lrp2−/− FVB/N and Lrp2−/− F1 samples compared with C57BL/6N (Fig. 4E). In the wild-type Lrp2+/+ comparison, we identified 20 differentially expressed cilia- and SHH-relevant genes, of which 11 were upregulated and 9 were downregulated in wild-type FVB/N and F1 embryos compared with C57BL/6N (Fig. 4J).
For functional analyses we selected top hits, based on the highest fold change and P-value identified in both the mutant and wild-type comparisons (Fig. 4E,J). Tubulin α 1C (Tuba1c) and Unc-51-like kinase 4 (Ulk4) were highly differentially expressed in a strain-dependent manner.

Tuba1c, a component of tubulin, was expressed at five times lower levels in FVB/N Lrp2−/− and wild-type Lrp2+/+ embryonic heads compared with both C57BL/6N genotypes (Fig. S4A). F1 samples also showed significantly lower expression levels than samples from the C57BL/6N strain (Fig. S4A). Tuba1c is listed in the database of ciliary genes (http://www.syscilia.org/goldstandard.shtml; Pusapati et al., 2018; van Dam et al., 2013) but data on its direct connection to the SHH pathway are limited. However, pathway analysis has linked Tuba1c to the SHH ‘off’ state (Fabregat et al., 2018; see also https://reactome.org/content/detail/R-HSA-5610787.1).

Ulk4, which belongs to a family of serine/threonine kinases, has been shown to regulate acetylation of α-tubulin, an important post-translational modification of microtubules (Lang et al., 2016). Ulk4 was expressed at five and two times higher levels in FVB/N and F1 genotypes, respectively, compared with C57BL/6N samples (Fig. S4A).

**ULK4 is a positive regulator of the SHH pathway localized to the primary cilium**

We next analyzed the candidate modifier genes in a context independent of LRP2 loss of function. To test whether Tuba1c or Ulk4 expression has functional effects on the SHH pathway, we used a dual luciferase reporter assay (Christ et al., 2012; Sasaki et al., 1997; Taipale et al., 2000; Zhang et al., 2006) and quantified luciferase levels compared with controls (Fig. 5A). Tuba1c is listed in the database of ciliary genes (http://www.syscilia.org/goldstandard.shtml; Pusapati et al., 2018; van Dam et al., 2013) but data on its direct connection to the SHH pathway are limited. However, pathway analysis has linked Tuba1c to the SHH ‘off’ state (Fabregat et al., 2018; see also https://reactome.org/content/detail/R-HSA-5610787.1).

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Identification of PTTG1 as a SHH pathway modifier

We next focused on highly regulated genes that had never hitherto been associated with the primary cilium and canonical SHH pathway, and went back to the unbiased set of DEGs. The transcript with the highest fold change value and most significant P-value was pituitary tumor transforming gene 1 (Pttg1), also known as securin (Fig. 4D,I). Higher Pttg1 transcript levels (Fig. S4A) found in rescue backgrounds were reflected at the protein level. Western analysis detected significantly higher amounts of PTTG1 protein in FVB/N and F1 total embryos compared with C57BL/6N samples (Fig. S4B). In addition, we demonstrated significantly higher immunofluorescence intensities for PTTG1 in the FVB/N and F1 forebrain neuroepithelium compared with C57BL/6N (Fig. S4C).

We next tested whether PTTG1 can influence SHH signaling capacity. Ptg1 overexpression in NIH-3T3 cells resulted in a significantly higher activation of the GLI luciferase reporter after SHH stimulation compared with control vector overexpression, and this was inhibited by KAAD-cyclopamine (Fig. 5A). Similar to Ulk4, Ptg1 overexpression had a stronger effect on SHH pathway activation under conditions of lower stimulation, using 1:20 diluted SHH-conditioned medium (Fig. 5A). Additionally, Ptg1 overexpression in hTERT RPE-1 cells significantly increased endogenous GLI1 levels after SHH stimulation (Fig. S5B), providing additional evidence for an enhancing effect of PTTG1 on SHH signaling strength.

We also performed knockdown experiments for PTTG1 in NIH-3T3 cells and hTERT RPE-1 cells. Silencing Ptg1 expression had a significant negative effect on endogenous Gli1 and GLI1 levels after SHH stimulation (Fig. S5E,G) and on SHH-responsive GLI-driven relative luciferase levels (Fig. 5B). Next, we assessed SHH-induced ciliary Smoothened (SMO) localization after silencing Ptg1 expression. SMO concentration in the cilium was significantly lower in Ptg1 siRNA-treated cells compared with controls after SHH stimulation (Fig. 5C–D’). Altogether, we observed clear and robust effects in PTTG1 gain-of-function experiments and a milder effect in our silencing experiments, where PTTG1 knockdown attenuated SHH pathway induction, but did not abolish SHH signaling.

We suggest that ULK4 and PTTG1, which are highly expressed in the FVB/N and F1 rescue backgrounds, are not necessary, but instead are enhancing components of the SHH machinery. This hypothesis is supported by our DESeq2 data and quantitative RT-PCR experiments, which showed a twofold increase in Gli1 levels in the FVB/N rescue strain (Fig. 5G).
Fig. 5. ULK4 and PTTG1 are positive modulators of the SHH pathway. (A,B) SHH-responsive GLI-Luciferase reporter assay on NIH-3T3 cells. Overexpression of B9d1, Ulk4 and Pttg1 resulted in a significant increase of relative luciferase levels compared with controls after SHH stimulation. Tuba1c overexpression showed no increase after SHH stimulation. NIH-3T3 cells were treated with medium containing SHH-Np or control medium. KAADCyclopamine was used to confirm the canonical SHH pathway response. Significance was assessed by two-way ANOVA; **P < 0.01, ***P < 0.001; ns, not significant. RLU, relative light units. Data are mean±s.d. (B) ULK4, PTTG1 and B9D1 knockdown (KD) significantly compromised SHH-induced pathway activation compared with stimulated controls in the luciferase assay. Significance was assessed using two-way ANOVA: ****P < 0.0001. Data are mean±s.d. (C-D) Knockdown experiments for ULK4, PTTG1 and B9D1 in NIH-3T3 cells. ARL13b (red, in C) and γ-tubulin (red, in D) immunostaining were used to visualize primary cilia and the base of the primary cilium, respectively. SHH induced ciliary Smoothened (SMO, green) immunocytochemistry in cells with control KD and candidate gene KD. DAPI (blue) marks nuclei. (C-D) Representative micrographs (C,D) and corresponding quantification (D‘) of endogenous SMO fluorescence intensity in the primary cilium. Data are mean±s.d. ****P = 0.0001 (one-way ANOVA). Boxed cilia are used for zoomed displays. Scale bars: 5 µm. (E) Confocal microscopy detected ULK4 immunostaining (green) in the cytoplasm of NIH-3T3 cells and in primary cilium (boxed), which are positive for acetylated tubulin (acet. tubulin, red). Scale bar: 2 µm. (F) Immunogold labeling of ULK4 in the forebrain neuroepithelium of wild-type C57BL/6N and FVB/N embryos at E9.5 showed clear localization of ULK4 at the microtubule-based axoneme in the shaft of the primary cilium (boxed), at the basal body of the cilium (arrowheads) and at the daughter centriole (arrow). ULK4 was also localized to the cytoplasm (asterisk). Scale bars: 100 nm. (G) DESeq2 normalized counts for Gli1 showed significantly higher expression level for Lrp2+/+ FVB/N compared with Lrp2+/+ C57BL/6N samples. Relative mRNA expression level analysis by qRT-PCR confirmed significantly higher Gli1 expression for Lrp2+/+ FVB/N compared with Lrp2+/+ C57BL/6N in E9.5 embryonic heads. Lrp2+/+ C57BL/6N, n=5; Lrp2+/+ FVB/N, n=4. Data are mean±s.d.
PTTG1 is a ciliary protein in the brain

We next analyzed the subcellular localization of PTTG1 and, as expected (Moreno-Mateos et al., 2011; Tong et al., 2008), we detected PTTG1 in the perinuclear region concentrated at the centrosome and in centrosomes of mitotic NIH-3T3 cells (Fig. 6A, arrow and arrowheads, respectively, and Fig. S6A, arrowheads). In some cells we also observed a dispersed PTTG1 signal in the cytoplasm (Fig. 6A, asterisk; Fig. S6A, asterisks). Interestingly, in non-mitotic NIH-3T3 cells, we detected PTTG1 in the primary cilium, a microtubule-based organelle, essential for SHH signaling. PTTG1 localized to a subset of primary cilia (Fig. 6B, arrowheads and Fig. S6B). We found PTTG1 in the periciliary region (Fig. 6C) as well as along the shaft of the primary cilium (Fig. 6D,E). Within the shaft PTTG1 protein localization was heterogenous, sometimes covering only the base or proximal region of the cilium (Fig. 6D and Fig. S6B,C) and in other cases the entire ciliary shaft (Fig. 6E). The variable localization of PTTG1 among different cilia suggested that it is not a structurally required component of the primary cilium, but might be shuttled into the shaft in a regulated fashion to support ciliary function. Ciliary localization for PTTG1 was confirmed in human hTERT RPE-1 cells (Fig. 6F), where PTTG1 was clearly localized to the shaft of the primary cilium. We further analyzed whether PTTG1 is also localized to cilia of the developing brain using cephalic explants from wild-type embryos (Fig. S7A). In neuroepithelial stem cells from both mouse strains, we detected PTTG1 localization to a subset of primary cilia (Fig. 7A, arrowheads; Fig. S7B) in the periciliary region (Fig. 7B,E) and in the shaft of the primary cilium labeled for ARL13b (Fig. 7C,D,F,G). Ultrastructural analysis revealed that PTTG1 was localized to the microtubule-based ciliary axoneme (Fig. 7H and Fig. S7C, boxed), to the periciliary region at the appendages (Fig. 7H, arrowhead and Fig. S7C, arrowheads) and to the basal body (Fig. 7H, arrow and Fig. S7C, arrow). PTTG1 localization at the base of and in the primary cilium is depicted in a graphical model (Fig. 7J). Taken together, our results identified PTTG1 as a microtubule-associated component of the primary cilium that can modulate efficiency of the canonical SHH signaling pathway.

Cilia number and length differ between neuroepithelial stem cells from C57BL/6N and FVB/N wild-type strains

Analyzing neuroepithelial stem cells from C57BL/6N and FVB/N cephalic explants revealed differences in cilia number per area (Fig. S7B) and in cilia length between the strains (Fig. 7I). Calculating the number of cilia in the anterior neural tube on the apical surface of the explants, we identified 645 cilia/mm² surface area in FVB/N compared with 425 cilia/mm² surface area in C57BL/6N explants (Fig. S7B). We also used super-resolution gated STED microscopy to image anterior neural tube explants from E9.5 C57BL/6N and FVB/N mice labeled for the regulatory GTPase ARL13b, which is localized to the membrane of the entire ciliary shaft (Fig. 7I). Strikingly, wild-type neuroepithelial stem cells of the rescue strain FVB/N had 20% shorter primary cilia, with an average length of 1.19±0.01 μm (s.e.m.) compared with the primary cilia of the HPE-susceptible strain C57BL/6N, with an average length of 1.49±0.014 μm (Fig. 7I).

DISCUSSION

Lrp2<sup>−/−</sup> mutant mice studied on a C57BL/6N, a mixed C57BL/6N; 129/SvEMS-Ter, a CD1 and a C3H/HeNcrl background show fully penetrant forebrain defects and perinatal lethality (Sabatino et al., 2017; Spoelgen et al., 2005; Willnow et al., 1996). We and others previously found that a Lrp2<sup>267/267</sup> ENU mutant line on a
predominant FVB/N background survived to adulthood (Gajera et al., 2010; Zarbalis et al., 2004; Zywitza et al., 2018). These studies prompted us to perform more-rigorous studies using a pure congenic Lrp2−/− FVB/N line to analyze the molecular mechanisms underlying these rescue effects.

Here, we demonstrate that the FVB/N background rescued HPE, which results from defects in the induction of the ventral forebrain, and most importantly rescued the underlying SHH signaling defects. Additionally, the previously described heart outflow tract phenotype of Lrp2−/− C57BL/6N mice (Baardman et al., 2016; Li et al., 2015) did not manifest in Lrp2−/− FVB/N embryos. Thus, genetic background strongly modifies the severity of congenital forebrain and heart defects in LRP2-deficient mice. The mouse models, described in this study, reflect the highly variable clinical
PTTG1 is a ciliary component in neuroepithelial stem cells. (A) Confocal microscopy on the E9.5 C57BL/6N (n=6) and FVB/N (n=6) cephalic explants shows PTTG1 (red) in a subset of ARL13b-positive (green) primary cilia (arrowheads). Scale bars: 1 µm. (B-G) High-resolution confocal 3D imaging: PTTG1 is present in the periciliary region (B,E) and in the ciliary shaft (C,D,F,G). Top panel insets (B-G) show magnified cilia (outlined in B-G) with single signals for PTTG1 (left, outlining the cilium), ARL13b (middle) and merge (right). Bottom panel insets (B-G) show the same cilia as follows: reconstruction of ARL13b with non-reconstructed PTTG1 signal (left), optical section through this (middle) and reconstruction of both ARL13b and PTTG1 (right). Scale bars: 1 µm. (H) Immunogold labeling of PTTG1 in the forebrain neuroepithelium of E9.5 embryos showed clear localization of PTTG1 at the microtubule-based axoneme in the shaft of the primary cilium. PTTG1 was also localized to the daughter centriole (arrow). PTTG1 was also detected in the periciliary region (arrowhead) and a few signals were detected in the cytoplasm (asterisk). Scale bars: 100 nm. (I) Differences in primary cilia length comparing C57BL/6N and FVB/N neuroepithelial stem cells. Primary cilia, immunolabeled for ARL13b (blue), were imaged in the anterior region of E9.5 cephalic explants using STED microscopy. Primary cilia of Lrp2+/+ FVB/N embryos (n=7) were 20% shorter [1.19±0.011 µm (s.e.m.); n=908] compared with cilia from Lrp2+/+ C57BL/6N (n=8) samples [1.49±0.014 µm (s.e.m.; n=1068). Cilia length was measured as indicated by red dashed lines. The box plot represents mean cilia length with whiskers indicating minimal and maximal values; unpaired t-test for statistical analysis; ***P<0.001. Scale bars: 1 µm. (J) Schematic of the primary cilium with components relevant to the present work. PTTG1 and ULK4 are ciliary components, localized to the periciliary region and the ciliary shaft.

Identification of modifier genes in humans is challenging. It is also difficult to distinguish between the impact of modifier genes and environmental factors on the clinical variability. Comparing LRP2-deficient embryos on a susceptible C57BL/6N and a resistant FVB/N mouse strain allowed us to identify disease-relevant candidate modifiers. Background modifier studies in early embryonic development, where the FVB/N strain was analyzed, are rare and could yet shed important light on the etiology of neural tube defects as reviewed by Leduc et al. (2017). Exencephaly in Cecr2 mutant mice shows strain-specific differences in penetrance, comparing BALB/cCrI and FVB/N, with the latter being a rescue background. Whole-genome linkage analysis revealed chromosome 19 as a modifier locus (Banting et al., 2005; Davidson et al., 2007; Koosstra et al., 2011).

In our study, we combined complex transcriptome and functional analyses to identify disease-relevant candidate modifiers. As we had documented a full rescue of HPE in FVB/N and F1, suggesting a dominant effect of FVB/N alleles, candidate modifier genes should be found in the overlapping pool of strain-specific DEGs, comparing C57BL/6N versus FVB/N and C57BL/6N versus F1 (Fig. 4C,H).

We identified Tubal1 and Ulk4 as the top down- and upregulated DEGs, respectively, among the unfiltered DEGs (Fig. 4D,I) and among the hedgehog pathway/cilia gene-filtered DEGs (Fig. 4E,J). Functional data on Tubal1 regarding ciliogenesis or SHH pathway regulation are very limited. In the Reactome Pathway Knowledgebase, Tubal1 is linked to the hedgehog (HH) ‘off’, state suggesting that this tubulin plays a role as a negative regulator in the SHH pathway (Fabregat et al., 2018; see also https://reactome.org/content/detail/R-HSA-5610787.1).

Ulk4 is postulated to play an essential role in brain development and has been genetically linked to increased susceptibility in manifestation of HPE that is observed even among family members carrying the same pathogenic gene variant (Heussler et al., 2002; Hong and Krauss, 2018; Krauss, 2007; Muenke and Beachy, 2000; Roesler et al., 2018).

In an unbiased approach, we asked whether the other top regulated DEG, Pttg1 (Fig. 4D,J), could potentially also affect SHH activity. PTTG1 is a known substrate for the anaphase-promoting complex (APC) and associates with separin until activation of the APC (Hagting et al., 2002; Mei et al., 2001; Thornton and Toczyski, 2003; Yanagida, 2000). Various functions for PTTG1 have been described, including control of mitosis, DNA repair, transcriptional activity and cell migration (Genkai et al., 2006; Havens and Walter, 2011; Hellmuth et al., 2020; Holt et al., 2008; Xiang et al., 2017; Yan et al., 2015; Zheng et al., 2015). The protein has tumorigenic activity and the gene is amplified in various human tumors of the breast, uterus, lung and thyroid (Bernal et al., 2002; Mirandola et al., 2015; Read et al., 2017; Vlotides et al., 2007; Zou et al., 1999). High expression of Pttg1 has also been associated with aggressive forms of brain tumors, including medulloblastoma and glioblastoma (Salehi et al., 2015; Yan et al., 2015). During embryonic development Pttg1 expression has been reported in the human and murine brain (Boelaert et al., 2003; Karsten et al., 2003; Tarabykin et al., 2000). A recent study demonstrated a role of PTTG1 in microtubule nucleation (Moreno-Mateos et al., 2011). However, PTTG1 has hitherto not been associated with microtubule-based axoneme function of the primary cilium or with canonical SHH pathway modulation in the cilium. We show that Pttg1 overexpression can indeed modulate SHH responsiveness (Fig. 5A and Fig. S5A,B). Therefore, we conclude that PTTG1 is a SHH pathway component and can promote canonical SHH signaling efficiency. We also discovered a previously unidentified and seemingly dynamic localization of PTTG1 to the periciliary region and to the microtubule-based axoneme of the cilium in NIH-3T3 and in neuroepithelial cells of the developing brain. The ciliary localization supports the hypothesis that PTTG1 modulates the SHH pathway via a cilia-associated mechanism. So far, only a relatively small number of regulatory proteins have been reported to be involved in both cytokinesis and ciliogenesis (Gromley et al., 2005; Kim et al., 2005; Pan et al., 2007; Park et al., 2008; Shah et al., 2008; Smith et al., 2011; Spektor et al., 2007; Verti et al., 2015; Zuo et al., 2009). The anaphase-promoting complex (APC), which is important for cytokinesis, is found to be also localized to the basal body of the primary cilium during interphase, where its activity regulates disassembly of the primary cilium (Wang et al., 2014).

PTTG1 has been genetically linked to increased susceptibility in humans to developing schizophrenia (Lang et al., 2014). Mice with Ulk4 gene defects show hydrocephaly, dilated brain ventricles and ependymal motile ciliary defects (Liu et al., 2016). Motile cilia defects in ULK4-deficient mice are characterized by defects in basal body orientation and axonemal organization, suggesting an important role for ULK4 in ciliary function (Liu et al., 2016). However, ULK4 function has not been associated with the SHH pathway or the primary cilium before. Here, we demonstrate a previously unknown function for ULK4 as it can enhance canonical SHH signaling capacity in a heterologous system (Fig. 5A and Fig. S5A,B) and is present in the primary cilium with a prominent signal in the basal body (Fig. 5F). Downregulation of ULK4 attenuated SHH-induced pathway activation and impaired ciliary SMO accumulation (Fig. 5B-D and Fig. S5E-G). The exact mechanism of how ULK4 modifies SHH pathway activity at the primary cilium still needs to be identified. It is hypothesized that ULK4 regulates neuronal function by acetylation of α-tubulin, an important post-translational modification of microtubules (Lang et al., 2014, 2016). It is therefore feasible to hypothesize that ULK4 is required for efficient microtubule nucleation at the basal body during ciliogenesis. Altogether, our data suggest that ULK4 might regulate the penetrance of SHH-related congenital disorders.

In a recent study, a role of PTTG1 in microtubule nucleation (Moreno-Mateos et al., 2011). However, PTTG1 has hitherto not been associated with microtubule-based axoneme function of the primary cilium or with canonical SHH pathway modulation in the cilium. We show that Pttg1 overexpression can indeed modulate SHH responsiveness (Fig. 5A and Fig. S5A,B). Therefore, we conclude that PTTG1 is a SHH pathway component and can promote canonical SHH signaling efficiency. We also discovered a previously unidentified and seemingly dynamic localization of PTTG1 to the periciliary region and to the microtubule-based axoneme of the cilium in NIH-3T3 and in neuroepithelial cells of the developing brain. The ciliary localization supports the hypothesis that PTTG1 modulates the SHH pathway via a cilia-associated mechanism. So far, only a relatively small number of regulatory proteins have been reported to be involved in both cytokinesis and ciliogenesis (Gromley et al., 2005; Kim et al., 2005; Pan et al., 2007; Park et al., 2008; Shah et al., 2008; Smith et al., 2011; Spektor et al., 2007; Verti et al., 2015; Zuo et al., 2009). The anaphase-promoting complex (APC), which is important for cytokinesis, is found to be also localized to the basal body of the primary cilium during interphase, where its activity regulates disassembly of the primary cilium (Wang et al., 2014).
Our data provide evidence that PTTG1, besides its function as a cell cycle regulator associated with the centrosome, also plays a role in ciliogenesis and ciliary function. We propose that higher expression levels of PTTG1 predispose neuroepithelial stem cells in FVB/N mice to more efficient SHH signaling by enhancing microtubule repolymerization of the ciliary axoneme after cell division (Moreno-Mateos et al., 2011). Knockdown of PTTG1 has been shown to attenuate microtubule repolymerization after nocodazole treatment, which leads to microtubule depolymerization (Moreno-Mateos et al., 2011). Efficient microtubule repolymerization after cell division is crucial for assembly of the primary cilium and therefore SHH signaling (He et al., 2017). In our study, enhanced ciliogenesis is also supported by the on average higher number of cilia per area in the FVB/N neuroepithelium compared with C57BL/6N (Fig. S7B).

Interestingly, all three top regulated candidate modifier genes, *Tuba1c*, *Ulk4* and *Pttg1*, identified in our screen, are linked to microtubule nucleation and function, suggesting that the expression profile of these genes in a FVB/N background provides an advantageous environment for the microtubule-based ciliary function and SHH signaling, and ultimately a protective effect against developmental defects.

ULK4 and PTTG1 are not necessary for establishing SHH signaling as our silencing experiments attenuated, but did not abolish, SHH signaling. Additionally, *Ulk4* and *Pttg1* null mutant mice do not display phenotypes related to a complete loss of SHH activity (Liu et al., 2016; Wang et al., 2001). However, ULK4 and PTTG1 have positive effects on the overall efficiency of the pathway and can enhance SHH signaling strength, as shown in this article. Expression profiles of these candidate modifier genes and of other still functionally uncharacterized modifier candidates most likely ensure sufficient SHH activity in the absence of LRP2 on a FVB/N background.

Differences in ciliary factors between the strains could also be reflected in different morphology of primary cilia. Indeed, our super-resolution imaging revealed striking differences in the morphology of primary cilia between C57BL/6N and FVB/N neuroepithelial stem cells (Fig. 7I), suggesting that the genome of wild-type mice from different mouse strains has a substantial influence on the shape of primary cilia. This could have functional consequences and relevant clinical implications, considering the complex signaling function of the primary cilium (Gigante and Caspary, 2020; Goetz and Anderson, 2010; Nachury and Mick, 2019; Singla and Reiter, 2006). Little is known about the regulation of cilia length and the implication of variable cilia length in health and disease, including ciliopathies (Fliegauf et al., 2007; Gerdes et al., 2009). Interactions of ciliary components, e.g. prominin with ARL13b, have been shown to play a role in maintenance of length of the primary cilium throughout the animal kingdom (Jászai et al., 2020). Studies in mammalian cells suggest that increased soluble tubulin production leads to longer cilia and cytosolic tubulin stabilization results in shorter cilia (Sharma et al., 2011; Wang and Dynlacht, 2018; Wang et al., 2013). Wang et al. showed that the complex formed by cell cycle regulator APC and its co-activator Cdc20 at the basal body maintains optimal ciliary length and is crucial to shorten the cilia when cells exit from quiescent stage (Wang et al., 2014). As PTTG1 is a substrate for APC (Mei et al., 2001; Yanagida, 2000), and also found at the base of the cilium in our study, this conceptually links the function of PTTG1 to cilia length regulation.

We conclude that the FVB/N inbred mouse strain has advantageous ciliary composition and morphology, and we identify plausible molecular candidates that render the strain more resilient to disturbances of the SHH pathway during early development (Fig. 8). Our study highlights the importance of mouse strain-dependent penetration of phenotypes and the role of modifier genes. As much as the FVB/N strain appears to be more resilient to forebrain patterning defects, as shown in this study, and to neural tube closure defects (Leduc et al., 2017), it is also commonly known that the FVB strain is more susceptible to mammary tumors whereas the C57BL/6 strain is more resistant (Davie et al., 2007). It will be interesting to test whether *Ptg1* and *Ulk4*, as SHH pathway stimulators, could be modifier genes that also modulate the mammary tumorigenesis in mouse models of breast cancer.

The expression profiles that differ profoundly between various wild-type strains during early developmental stages provide a unique resource that, to our knowledge, has not been available before. Considering the huge effects of the FVB/N background, this resource is particularly valuable to understand genetic robustness in brain and heart development. Furthermore, we show that genetics in the mouse combined with functional cell biological studies can define novel molecular mechanisms that potentially underlie variability in the penetrance of human cilia-associated neurodevelopmental disorders. Identification of disease relevant modifier genes in the mouse can provide important insights into the etiology and prevention of human congenital disorders.
MATERIALS AND METHODS

Animals
Experiments involving animals were performed according to institutional guidelines following approval by local authorities (X0005/12). Mice were housed in a 12 h light-dark cycle with ad libitum food and water. The generation of mice with targeted disruption of the Lrp2 gene has been described before (Willnow et al., 1996). The Lrp2 mutant mouse line was crossed onto a pure C57BL/6NCrl background in our laboratory (Slopen et al., 2005) (herein referred to as Lrp2 mutant C57BL/6). For this study, the Lrp2+/− C57BL/6N line was backcrossed for more than 12 generations to obtain a congenic mouse line on a FVB/NCr1 background (herein referred to as Lrp2 mutant on FVB/N background).

We used mice over 8 weeks of age for timed matings for our experiments. Analyses of the congenital defects were carried out in Lrp2+/− and in somite matched Lrp2+/−/+ and Lrp2−/−−/+ control littermates on a C57BL/6NCrl and FVB/NCr1 background. For the generation of the F1 hybrid Lrp2 mutant embryos, which were used for RNA sequencing experiments, Lrp2+/− FVB/NCr1 females were crossed with Lrp2+/− C57BL/6NCrl males. For phenotype analyses on F1 Lrp2 mutant embryos, Lrp2+/− FVB/NCr1 females were crossed with Lrp2+/− C57BL/6NCrl males to generate FVB86F1 embryos and, conversely, to generate B6FVB81 embryos. No difference in the phenotypic appearance was observed between these Lrp2+/− F1 mutants. PCR genotyping was performed from yolk sac genomic DNA for E8.5 embryos and from tail biopsies for older embryos.

Histology
Standard Nissl and Hematoxylin and Eosin stainings were performed on paraffin wax-embedded sections. Embryos were fixed in 4% paraformaldehyde in PBS at 4°C, overnight, embedded in paraffin wax and cut at 10 μm. Nissl staining was performed in 0.1% Cresol Red solution (Sigma Aldrich, 114480) for 3-10 min. Hematoxylin and Eosin staining was performed according to manufacturer’s instructions with incubation times of 5 min in Hematoxylin (Carl Roth, T865) and 3 min in Eosin (Carl Roth, X883.2). Sections were dehydrated and mounted in Roti Histokit (Carl Roth, 6638.1). Staining was visualized using the Leica DM5000B microscope with LAS-X 3.3.3 software.

In situ hybridization
Whole-mount in situ hybridization was carried out as described previously (Hamnes et al., 2001). In situ hybridization on sections was performed as described previously (Jensen and Wallace, 1997), except that the signal was enhanced by performing the color reaction in the presence of 10% poly vinyl alcohol (Sigma Aldrich, P8136). Probe synthesis was conducted with the components of the DIG RNA labeling kit (Roche, 11277073910). Plasmids for generating in situ probes were generated from the following mRNA sequences: Nkx2.1 (NM_009385.3, bp 2032-2813) kindly provided by Carmen Birchmeier (MDC, Berlin, Germany) and Shh (NM_009170.3, bp 455-1097) kindly provided by Andrew P. McMahon (University of Southern California, Los Angeles, USA). Images of embryonic heads were taken using a Leica MZ 10F stereomicroscope (Leica LAS V4.9 imaging software). Images were processed with the Adobe Photoshop select and mask tool to identify embryonic heads and adjust the background color.

Labeling of heart samples with Batson’s pigment
Polymeric dye injections were used to visualize the ascending aorta and the pulmonary artery in isolated E18.5 mouse hearts applying the Batson’s #17 Anatomical Corrosion Kit (Polyscience, 07349). Batson’s #17 Blue pigment was added to Base Solution A at 2%, mixed vigorously and divided into two equal parts. 24 ml of the Cataly whole solution was added to the first 100 ml of Base Solution A/pigment mix. 24 drops of Promoter C were carefully added to the second half of the Base Solution A/pigment and mixed slowly. Both solutions were mixed together and stirred. After both parts were mixed, it took 30-45 min until the solution was fully polymerized and until then the experiment was finished. The same procedure was repeated for Batson’s #17 Red pigment. Injections of the blue and red pigment solution into the right and left ventricle, respectively, were made with a disposable polyethylene syringe and 23 G needle under a stereomicroscope (Leica MZ 10F). Images of the heart were taken after the injection (Leica LAS V4.9 imaging software).

RNA library generation and sequencing
For the generation of an RNA library, embryonic heads of E9.5 embryos were dissected, snap frozen and stored at −80°C until RNA isolation. RNA was extracted using RNeasy Plus Micro kit (Qiagen, 74034), checked on Bioanalyzer (Agilent RNA 6000 Nano kit, 5067-1511) and samples with RIN>9 were used to prepare the cDNA library. Library preparation for mRNA sequencing was performed according to the Illumina SR TruSeq Stranded mRNA protocol (Cluster Kit V3, Illumina, 20020594) on 23 embryonic head RNA samples from mice with 24 somites. Sequencing was performed on the Illumina HiSeq2000 system with HCS 2.2.38 software.

Sequencing reads were aligned to a SNP-infused (FVB/NCr1 and C57BL/6NCrl SNPs, accordingly) mouse genome (Ensembl GRCm38.77) using TopHat v2.0.12 with Bowtie 2.0.6. The number of reads that mapped to a gene was counted using the HTSeq-count program with default parameters.

Allelic expression analysis was determined by counting the reads matching the C57BL/6N or FVB/N genotype in the F1 mRNA-seq data.

Differential gene expression analysis
mRNA-seq quantifications were derived from exon-mapped, paired-end reads. Expression quantification was followed by read normalization, size factor estimation and differential expression analysis using DESeq2 v1.12.4 (Love et al., 2014). We defined genes as differentially expressed if they met our significance threshold of FDR ≤0.05. Allele-specific expression was determined by counting the reads matching the C57BL/6N or FVB/N genotype in the F1 mRNA-seq data.

Real-time quantitative reverse transcription PCR (real-time qRT-PCR)
Total RNA from E9.5 embryos heads was isolated using RNeasy Plus Micro kit (Qiagen, 74034). RNA from NIH-3T3 and hTERT RPE-1 cells was extracted using TRizol Reagent (Thermo Fisher, 15596026). cDNA was synthesized by high-capacity RNA-to-cDNA kit (Thermo Fisher, 4387406). Quantitative PCR was performed using TaqMan Universal PCR Master Mix (Thermo Fisher, 4304437) with the BioRad CFX384 Real Time System used on a BioRad C1000 Touch Thermal Cycler. The following TaqMan probes were used: Gli1 (mouse, Thermofisher, assay ID: Mm00494654_m1), Prtg1 (mouse, Thermofisher, assay ID: Mm04954658_m1), PTTGI (human, Thermofisher, assay ID: Hs00851754_v1) and ULK4 (human, Thermofisher, assay ID: Hs00289085_m1). The expression of genes was normalized to Gapdh (mouse, Thermofisher, assay ID: Mm9999915_g1) or GAPDH (human, Thermofisher, assay ID Hs99999905_m1). Transcript levels relative to Gapdh were calculated using the deltaCt method. Data were analyzed in GraphPad Prism 7 using an unpaired t-test or ANOVA. The method is indicated in the figure legends.

Cell culture
The NIH-3T3 and HEK293T cell lines were obtained from T. Willnow (MDC, Berlin, Germany) (Christ et al., 2012). SHHIN-293 cells were originally kindly provided by M. Kato (Stanford School of Medicine, CA,
Cells were maintained in DMEM (Invitrogen, 31966047) with 10% fetal bovine serum (FCS, PAN-Biotech, P40-37500) and 1% penicillin-streptomycin (Invitrogen, 15401022). Human cell line hTERT RPE-1 (ATCC CRL4000) was purchased from the American Type Culture Collection (ATCC, 70021355). Cells were maintained in DMEM: F12 Medium (ATCC 302006) with 10% fetal bovine serum (FCS, PAN-Biotech, P40-37500) containing 0.01 mg/ml hygromycin B (Santa Cruz, sc-506168). All cell cultures were free of contamination. hTERT RPE-1 cells were purchased from ATCC in July 2020 and certified to be free of mycoplasma contamination.

For overexpression experiments, NIH-3T3 cells were transfected with Lipofectamine 3000 Transfection Reagent (Invitrogen, L3000005) according to the manufacturer’s instructions. The following overexpression constructs were used: cDNA ULK4 (Origene, MR202008), cDNA ULK4 (Origene, MR217918), B9d1 RIKEN cDNA clone (Source Bioscience, 07100008G05), Tubal: RIKEN cDNA clone (Source Bioscience, 2810407E13). Following 24 h transfection, NIH-3T3 cells were stimulated for 48 h with conditioned medium from HEK293 cells stably secreting SHH-Np (SHHN-293 cells; kindly provided M. Kato, Stanford School of Medicine, CA, USA) or control medium from parental HEK293T cells, at a 1:10 dilution, unless stated otherwise. The hTERT RPE-1 cells were transfected with TransIT-LT1 Transfection Reagent (Mirus, MR2304) according to the manufacturer’s instructions. 24 h after transfection, hTERT RPE-1 cells were serum starved for 24 h to promote cilia formation and then stimulated for 48 h with conditioned medium from HEK293 cells stably secreting SHH-Np or control medium, at a 1:10 dilution.

ULK4, PTTG1 and B9D1 silencing was achieved by siRNA transfection. NIH-3T3 or hTERT RPE-1 cells were transfected using Lipofectamine RNAiMAX (Thermo Fisher, 13778-150) for the western blot and/or qPCR experiments. NIH-3T3 cells were transfected using DharmaFECT Duo Transfection Reagent (Hank, T-2010-02) for the dual luciferase reporter assay. The following siRNA was used at final 50 nM concentration in NIH-3T3 cells: ULK4 (Thermo Fisher, AM16708, Assay ID: 168185), Pttg1 (Thermo Fisher, 4390771, Assay ID: s78255), B9d1 (Thermo Fisher, 4390771, Assay ID: s77534) and negative control (Thermo Fisher, 4390847). The following siRNA was used at final concentration of 40 nM in hTERT RPE-1 cells: ULK4 (Thermo Fisher, 4390824, Assay ID: s29923), PTTG1 (Thermo Fisher, 4390820, Assay ID: s17653). Prior to the final assay, cells were subjected to the SHH stimulation with conditioned medium using the same method as for overexpression experiments described above. The knockdown was validated using western blot or qPCR (see Fig. S5).

**Dual luciferase reporter assay**

NIH-3T3 cells, seeded in 24-well plates, were transiently co-transfected with the gene of interest or a completely empty plasmid, GLI-dependent firefly luciferase reporter (8×3 Gli-BsS565LucI) (Sasaki et al., 1997) and a constitutive Renilla luciferase reporter (pRL-TK; Promega, E2241) at a ratio of 10:1:1. In the knockdown experiments, 50 nM siRNA targeting gene of interest or a negative control siRNA was used. 24 h later, the medium was replaced with conditioned medium from HEK293 cells stably secreting SHH-Np (SHHN-293 cells; kindly provided M. Kato, Stanford School of Medicine, CA, USA) or control medium from parental HEK293T cells, at a 1:10 dilution or 1:20 dilution (low SHH), in medium containing 0.5% FCS. For the control studies KAAD-cyclopanmine (Calbiochem, 239804) at a 50 nM concentration or solvent was added to the conditioned medium with SHH-Np.

After 48 h of stimulation, activity was assayed with D-luciferin (ZellBio, LUCK-100) and results were normalized to the corresponding Renilla activity, assayed with coelenterazine (ZellBio, C2Z-5). For Dual Luciferase reporter assay measurements, a Centro XS® LB 960 Microplate Luminometer (Berthold Technologies) and MikroWin 2000 4.41 software (Mikrotek Laborsysteme) was used. Experiments were performed in triplicate, referring to three different wells assayed the same day, in minimum of three independent experiments; results are shown as mean±s.d. Statistical analyses were performed using two-way ANOVA with Dunnett’s multiple comparisons test.

**Western blot analysis**

E8.5 whole embryos were lysed in SDS lysis buffer [60 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.01% Bromophenol Blue and 1.25% β-mercaptoethanol]. Samples were heated to 95°C for 5 min, centrifuged for 2 min at 16,363 g and stored at −20°C until used. Cells were lysed in the RIPA lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate and 1 mM AEBSF]. Equal amounts of samples were subjected to a Tris-Glycine Gel (Invitrogen, XP0012C) in a Mini Gel Tank (Invitrogen, A25977). The resolved proteins were transferred onto a nitrocellulose membrane (Amer sham Pro tran 0.2 μm, 106000006) using a wet electroblotting system (Bio-Rad Mini Protein II Cell) followed by immunoblotting. For GLI1 detection, transfer without methanol was performed. 5% non-fat dry milk in TBS-T (0.1% Tween-20) was used for blocking at room temperature for 1 h. Primary antibodies were used overnight at 4°C as follows: rabbit anti-PTTG1 (Abcam, ab79546, 1:5000, knockdown validated by Abcam and knockdown validated in this work, see Fig. S5), mouse anti-alpha Tubulin (Merck Millipore, CP06, 1:2000), mouse anti-GLI1 (Cell Signaling, 2643S, 1:1000), rabbit anti-HSP90 (Cell Signaling, 4874S, 1:1000), rabbit anti-ULK4 (Novus Biologicals, NBPI-20229, 1:2000, knockdown validated in this work, see Fig. S5 and Lang et al., 2014) and mouse anti-GAPDH (Santa Cruz, sc23223, 1:5000). Signal was detected by SuperSignal West Dura (Life Technologies, 34075) with an Optimax 2010 X-Ray Film Processor (PROTECT) or using the BioRad ChemiDoc MP Imaging System. The results were quantified using ImageJ, with one-way ANOVA or unpaired t-test as a statistical analysis.

**Sample preparation for confocal microscopy**

**Immunofluorescence on sections**

Standard immunofluorescence was performed on cryo- and paraffin sections. For cryosections, PFA fixed embryos were infiltrated with 15% and 30% sucrose in PBS for up to 24 h depending on the stage, embedded in OCT (Tissue-Tek, Sakura Finetek, sc-4583) and cut into 10 μm coronal sections. For paraffin wax-embedded sections PFA fixed embryos were dehydrated, incubated in RotiHistol (Carl Roth, 6640, 1:100) and embedded in paraffin wax and cut into 10 μm sections. Standard immunohistochemical analysis was carried out by incubation of tissue sections with primary antibodies at the following dilutions: rabbit anti-SHH (Santa Cruz, sc9024, 1:50), sheep anti-LRP2 antisera (1:4000; kindly provided by the Laboratory of Renata Kozyraki, INSERM, University of Paris, France) rabbit anti-LRP2 (Abcam, ab76969, 1:1000) and rabbit anti-PTTG1 (Abcam, ab79546, 1:100, knockout validated by Abcam). Bound primary antibodies were visualized using secondary antisera conjugated with Alexa Fluor 488, 555 and 647 (1:500, Abcam). All samples were counterstained with DAPI (Invitrogen, 62248). Sections were mounted with Dako Fluorescence Mounting Medium (Agilent, S302380-2).

**Immunocytochemistry**

For immunocytochemistry, NIH-3T3 cells were seeded in chamber slides (Sigma, C6807) in regular 10% FCS/DMEM medium conditions. After 24 h, cells were rinsed with 1×PBS fixed with 4% PFA for 15 min at room temperature and permeabilized with PBS-TritonX 0.25% for 20 min. Blocking with 10% donkey serum/PBS-TritonX for 1 h, was followed by a standard incubation with primary and secondary antibodies, as described above. Primary antibodies were used at the following dilutions: rabbit anti-SHH (Santa Cruz, sc9024, 1:50), sheep anti-LRP2 antisera (1:4000; kindly provided by the Laboratory of Renata Kozyraki, INSERM, University of Paris, France) rabbit anti-LRP2 (Abcam, ab76969, 1:1000) and rabbit anti-PTTG1 (Abcam, ab79546, 1:100, knockout validated by Abcam). Bound primary antibodies were visualized using secondary antisera conjugated with Alexa Fluor 488, 555 and 647 (1:500, Abcam). All samples were counterstained with DAPI (Invitrogen, 62248). Sections were mounted with Dako Fluorescence Mounting Medium (Agilent, S302380-2).
For immunocytochemistry, hTERT RPE-1 cells were seeded on cover slips (Paul Marienfeld, 0111520). After 48 h, cells were rinsed with 1×PBS and fixed in 4% PFA for 15 min at room temperature. Permeabilization and antibody staining was performed as described for NIH-3T3.

Preparation of mouse cephalic explants for immunofluorescence analysis

Explants were prepared analogously as described previously (Echevarria et al., 2002). E9.5 mouse embryos were collected and the neural tube was cut open along the dorsal midline, from a caudal to rostral direction, using an insect needle. The neural folds were precisely cut above the heart and placed on the sterile filter (Millipore, MCEP06H48) on the Petri dish in the drop of 1×PBS. The floor plate at the level of the cephalic flexure was pinched in order to unfold the tissue with the ventricular part facing up. A filter was placed in the six-well plate containing DMEM/10% FCS and explants were incubated at 37°C, with 5% CO2 and 95% humidity for 3–4 h to flatten and recover. The explants were washed gently in 1×PBS, fixed 1 h in 4% PFA, and subjected to the standard immunofluorescence protocol described above, with 2 nights of primary antibody incubation, as follows: rabbit anti-PTTG1 (Abcam, ab79546, 1:100), goat anti-PTTG1 (LifeSpan Biosciences, LS-B5119-50, 1:100), mouse anti-ARL13b (UC Davis/NIH NeuroMab, 75–287, 1:500). Secondary antibodies conjugated with Alexa Fluor 488 were used to visualize primary cilia and secondary antibodies conjugated with Alexa Fluor 647 were used to visualize PTTG1 signals. Explants were flat mounted in Slow Fade Diamond Antifade Mountant (Invitrogen, S36963) using Secure-Seal Spacer (Invitrogen, S24737) in order to match the refractive index (RI) of the mountant and the immersion media of the glycerol objective, and to prevent distortions of the explant by pressure, shrinking and hardening of the mountant.

Confocal microscopy image acquisition

Image acquisitions of tissue sections were carried out using either a Leica SPE or Leica TCS SP8 confocal microscope using a HC PL Apo 20× NA 0.75 MultiIMM and HC PL Apo 63× NA 1.3 oil immersion objective. All samples that were compared either for qualitative or quantitative analysis were imaged under identical settings for laser power, detector and pixel size. NIH-3T3 cells were imaged with a Leica TCS SP5 or Leica TCS SP8 confocal microscope equipped with a ACS Apo 63× oil NA 1.3 immersion objective. hTERT RPE-1 cells were imaged with Zeiss LSM 700 confocal microscope equipped with a plan-Apo 63× 1.4 oil immersion objective. The forebrain region of the explant samples was imaged en face with a Leica TCS SP8 confocal microscope using a HC PL Apo 63× NA 1.3 glycerol immersion objective with a working distance of 0.3 mm to enable high-resolution imaging with minimal spherical aberrations of the thick specimen. Three regions per explant were imaged for n=6 C57BL/6N and n=6 FVB/N. High-resolution z-stack (60 nm pixel size, 12 bit, 0.2 µm z-stack size) images of apical side of neuroepithelium were acquired with a z-piezo stepper. In all samples, Alexa Fluor 488 was excited by a 488 nm laser, detection at 660–730 nm, and DAPI was excited at 405 nm, with a pinhole set to 1 AU.

Image processing and analysis

3D data processing, deconvolution and correction

Confocal z-stacks of explants were subjected to a background correction and processed by deconvolution with the CMLE algorithm in order to obtain an improved signal-to-noise ratio, and axial and spatial resolution using Huygens Professional 19.10 software (Scientific Volume Imaging). For optimal deconvolution, the experimental PSF was calculated with Huygens PSF Distiller by using sub-resolution TetraSpeck Microspheres, 0.2 µm (Invitrogen, T7280) and acquired and used in the same imaging conditions as the explant sample. The same deconvolved beads sample was used to estimate a chromatic aberration correction matrix (in x/y/z) which was used to correct the experimental sample data. To obtain isotropic voxel values, the aspect ratio of the deconvolved bead sample was changed. This new sampling value was applied to all image data with the IMARIS Software before carrying out further segmentation and analysis steps.

Immunofluorescence signal localization analysis

Localization of the protein of interest (PTTG1) within the primary ciliature of mouse neuroepithelium was assessed using IMARIS software (Imaris 9.3 and 9.5, Bitplane) after applying raw data corrections (deconvolution, chromatic shift correction and non-isotropic imaging correction). 3D rendering and surfaces reconstruction of individual cilia was performed for two channels – one for cilia marker (ARL13b) and one for PTTG1 – and the classical object-based colocalization approach (individual segmentation and voxel based colocalization) with IMARIS XT module was used. To visualize the precise spatial co-existence of the two proteins in individual cilia, views from different angles were created and transparent LUTs as well as clipping planes were used to cut the 3D surfaces open to enable visualization of inside the cilia. Total number of cilia in each sample was assessed during the same processing steps from the reconstructed surface of ARL13b signal, and was given as number of cilia per area (Fig. S7B).

Quantification of immunofluorescence signal intensity

Z-stack images of the coronal sections were analyzed using ImageJ (Fiji, NIH). For the quantification of PTTG1 signals in the neuroepithelium, the full z-stack was used. The region of interest (ROI) was manually outlined as shown in Fig. S4C and the mean fluorescent intensity was measured with the ROI manager. The average intensity for each animal was used in the final quantification and one-way ANOVA statistical analysis was performed to assess the significance.

For the ciliary SMO fluorescence intensity quantification, z-stack images from NIH-3T3 cells were subjected to the background subtraction and semi-automated threshold adjustment of SMO signals. The mean signal intensity was referred to as ciliary only when directly related to the γ-tubulin-positive ciliary base. The experiment was performed in triplicate referring to three separate wells. One-way ANOVA statistical analysis was performed.

Immunofluorescence analysis of primary cilia using STED microscopy imaging

En face single-color stimulated emission depletion (STED) microscopy imaging was performed on the mouse cephalic explants, which were prepared as described above, with the exception that the highly cross-absorbed secondary antibody Alexa Fluor Plus 594 (Invitrogen, A32744) and ProLong Gold Antifade mountant (Invitrogen, P36934) were used to obtain optimal resolution. STED microscopy images were taken with a Leica SP8 TCS STED microscope (Leica Microsystems) equipped with a pulsed white-light excitation laser (WLL; ~80 ps pulse width, 80 MHz repetition rate (NKT Photonics) and two STED laser for depletion at 592 nm and 775 nm. The system was controlled by the Leica LAS X software. Single-color STED imaging was performed by exciting Alexa Fluor Plus 594 at 594 nm and the depletion of its emission with the 775 nm STED laser. Time-gated detection was set from 0.3 to 6 ns. The fluorescence signal was detected from 604 to 650 nm by a hybrid detector (HyD) at appropriate spectral regions separated from the STED laser. Images were acquired with a HC PL APO CS2 100×/1.40 NA oil objective (Leica Microsystems), a scanning format of 1024×1024 pixels, 8-bit sampling, 16× line averaging and 6× optical zoom, yielding a pixel size of 18.9×18.9 nm. Additionally, for every STED image, a confocal image with the same settings but only 1× line averaging was acquired.

All STED microscope images of the cilia within each independent experiment were acquired with equal settings. Single optical plane images were subjected to the cilia length quantification in ImageJ. Regions of interest (ROIs) were manually selected with the segmented line tool and the total lengths of single cilia were measured. The average cilia lengths for both mouse lines were checked for the statistical significance using an unpaired t-test.

Scanning electron microscopy

For scanning electron microscopy, E8.5 embryos were dissected and fixed in 0.1 M sodium cacodylate buffer (pH 7.3/7.4) containing 2.5% glutaraldehyde. After rinsing in cacodylate buffer, a post-fixation step of 2% OsO2 for 2 h followed. Samples were dehydrated in a graded ethanol
Transmission electron microscopy and immunogold labeling

The head region of mouse embryos at E9.5 was fixed with 3% freshly made formaldehyde/0.05% glutaraldehyde (EM grade) in 0.1 M phosphate buffer for 1 h at room temperature. After washing, samples were infiltrated with 2.3 M sucrose overnight at 4°C and frozen. Semi-thin sections were prepared to identify the region of the neuroepithelium. Ultrathin 100 nm crossections according to Tokuyasu were labeled with knockout-validated anti-PTTG1 antibody (Abcam, ab79546, diluted 1:50) or anti-ULK4 antibody (Novus Biologicals, NB1P-20229, 1:300), and 12 nm colloidal gold anti-rabbit secondary antibody (Dianova, 111-205-144). Sections were contrasted and stabilized with a mixture of 3% tungstosilicic acid hydrate and 2.5% polyvinyl alcohol. EM pictures were taken at 80 kV with a Morgagni electron microscope (Thermo Fisher), equipped with a Morada camera and the iT EM software (EMIS, Münster, Germany).

Data availability

Raw RNA sequencing datasets have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB46911. LRP2 in cardiac development. Dis. Model. Mech. 9, 413-425. doi:10.1242/dmm.2022053

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Quantification and statistical analysis

Tests used to analyze the data were carried out using Prism 7 software (GraphPad) and are mentioned in the respective figure legends. Figures were prepared using Adobe Illustrator 2019 software.

The term significant was used if P<0.05. Exact P values, n numbers and biological replicates are reported in the figure legends, supplementary tables or appropriate section in the Materials and Methods.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: A.H.; Methodology: M.L., M.R., A.S., B.P., N.H.; Formal analysis: M.R.; Investigation: M.L., M.R., A.S., B.P., N.H.; Resources: N.H.; Writing - original draft: A.H.; Visualization: N.M., I.K.; Supervision: A.H.; Project administration: A.H.; Funding acquisition: A.H.

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