Sonic Hedgehog promotes proliferation of Notch-dependent monociliated choroid plexus tumour cells

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Aberrant Notch signalling has been linked to many cancers including choroid plexus (CP) tumours, a group of rare and predominantly paediatric brain neoplasms. We developed animal models of CP tumours, by inducing sustained expression of Notch1, that recapitulate properties of human CP tumours with aberrant NOTCH signalling. Whole-transcriptome and functional analyses showed that tumour cell proliferation is associated with Sonic Hedgehog (Shh) in the tumour microenvironment. Unlike CP epithelial cells, which have multiple primary cilia, tumour cells possess a solitary primary cilium as a result of Notch-mediated suppression of multiciliate differentiation. A Shh-driven signalling cascade in the primary cilium occurs in tumour cells but not in epithelial cells. Lineage studies show that CP tumours arise from monociliated progenitors in the roof plate characterized by elevated Notch signalling. Abnormal SHH signalling and distinct ciliogenesis are detected in human CP tumours, suggesting the SHH pathway and cilia differentiation as potential therapeutic avenues.

Choroid plexus (CP) neoplasms represent rare primary brain tumours found predominantly in children. CP papillomas (CPPs) are more common and benign, whereas CP carcinomas (CPCs) are relatively rare and malignant. These tumours are believed to originate from CP epithelium, which differentiates from the roof plate to form the CP, a specialized tissue that produces cerebrospinal fluid (CSF) in each ventricle of the brain. Surgical resection remains the primary treatment for CPPs and is associated with excellent prognosis. However, clinical outcomes for patients with incompletely resected tumours, recurrent tumours, metastatic spread, or CPCs can be devastating. NOTCH signalling, tumour protein p53 (TP53) mutations, and genetic and epigenetic changes have been described.

Sonic hedgehog (Shh) signalling, a crucial pathway in development and cancers, is mediated by Patched (Ptc1) and Smoothened (Smo) receptors in the primary cilium where they orchestrate a signalling cascade that activates the expression of downstream targets, including Gli1, Mycn and cyclin D1 (Ccn1; refs 16,17). By inducing sustained Notch1 expression, we developed mouse models of CP tumours that closely resemble human CP tumours with abnormal NOTCH signalling. We show that the proliferation of Notch-induced CP tumours relies on Shh from the tumour microenvironment through their primary cilium. Aberrant SHH signalling and unique cilia patterns found in human CP tumours may serve as potential therapeutic targets.

RESULTS
Notch pathway activation leads to CP tumours
A molecularly defined boundary exists between the rhombic lip consisting of neural progenitors expressing the transcription factor
Atonal homologue 1 (Atoh1), also known as Math1 and the roof plate, characterized by the expression of Wnt1, Gdf7 and the transcription factor Lmx1a (ref. 18–21). Some Lmx1a+ cells are present in the rhombic lip and contribute to the cerebellum20,21. To determine whether rhombic lip progenitors contribute to the roof plate/CP lineage, we used Math1-Cre to drive Cre expression in Atoh1+ progenitors22 (Fig. 1a). When crossed with the Rosa26–EYFP Cre reporter strain23, the resulting Math1–Cre;Rosa26–EYFP mice have cells expressing enhanced yellow fluorescent protein (EYFP) in the CP in addition to the cerebellum (Fig. 1b). Although these EYFP+ cells comprise <0.5% of hindbrain CP epithelium, they express CP markers Lmx1a, orthodenticle homeobox 2 (Otx2), cytokeratins and aquaporin 1 (Aqp1; Fig. 1c,d and Supplementary Fig. 1a), indicating that some Atoh1+ progenitors contribute to hindbrain roof plate/CP lineage.

To determine the effects of Notch signalling on CP, Math1–Cre;Rosa26–EYFP mice were crossed with a Nicd1–Ires-GfpR26 strain that expresses Cre recombinase under the control of the Wnt1 enhancer24. Nicd1Cre;Rosa26–EYFP animals contain the human papilloma (CPP) and normal CP. Vesicular sac with accumulated CSF is shown (asterisk). Scale bars, 25 μm (white) and 500 μm (black). (f) H&E staining of human CP papilloma (CPP) and normal CP. Scale bar, 25 μm. (g) qRT–PCR analysis of Hes1 and Hes5 expression in CP tumours (black circles) and wild-type CPs (white circles) at P0, P7, P14 and P21 (data from technical replicates of each specimen set in a single experiment are shown; experiment was not repeated; raw data are available in Supplementary Table 9). (h) The expression of Ki-67 is shown in CP tumours from Mcre:Nicd1 mice, Lmx1a–Cre;Rosa26–Nicd1 (Lcre;Nicd1) mice, and normal CPs from wild-type mice. Dotted lines mark the boundary of lateral ventricles. The expression of Ki-67 in human CPP and normal CP is shown. Scale bars, 50 μm.
conditionally expresses the intracellular domain of Notch1 (NICD1) and green fluorescent protein4 (GFP, Fig. 1a). In Math1-Cre;Rosa26-NICD1;Rosa26-EYFP animals, hindbrain CP is significantly enlarged with many EYFP+ cells (Fig. 1b). Sustained NICD1 expression in Atoh1 lineage leads to >50-fold increase (~50%) in its contribution to hindbrain CP epithelium at birth that peaks at postnatal day

Figure 2 Notch-induced CP tumours undergo enhanced proliferation and exhibit defects in differentiation. (a) The expression of Ki-67 is shown in CP tumours from Mcre;NICD1 mice at P0, P7 and P14; CPs from P0 wild-type (WT) and Mcre;EYFP mice serve as the control. Ki-67 expression (red) labels proliferating cells, GFP expression (green) marks NICD1+ tumour cells, expression of Lmx1a (green) labels CP epithelium in wild-type mice, and EYFP expression (green, arrowheads) detected with a GFP antibody demarcates CP epithelial cells derived from Atoh1+ progenitors in Mcre;EYFP mice. DAPI staining (blue) marks nuclei. Scale bar, 25 μm. (b) Quantification of the percentage of Ki-67+ cells in NICD1+GFP+ tumour cells from Mcre;NICD1 mice and Lmx1a+ wild-type CP epithelial cells at different postnatal day (P) time points (n=3 CPs from three animals per genotype per time point, data from a single experiment are shown, raw data are available in Supplementary Table 9; mean ± s.e.m., two-way ANOVA, ****P<0.0001). (c) The expression of Ki-67 and Cdkn1b (p27 Kip1) is shown in tumours from Mcre;NICD1 mice and wild-type (WT) CPs at P7. Note that staining for Ki-67 and p27 is mutually exclusive, and p27 or Ki-67 expression is absent in wild-type CP epithelium despite positive staining in the cerebellum (Cb). Scale bar, 25 μm. (d) The expression of Lmx1a (red), Otx2 (red), Ttr (red), Aqp1 (red) and cytokeratins (red) is shown in CP tumours. GFP expression (green) labels NICD1+ tumour cells. Dotted lines mark the boundary between GFP+ tumour cells and epithelium. DAPI staining (blue) marks nuclei. Scale bar, 25 μm. (e) qRT–PCR analysis of Ttr and Aqp1 expression in tumours from Mcre;NICD1 mice (black circles) and wild-type (WT) CPs (white circles) at different time points (data from technical replicates in a single experiment are shown and available in Supplementary Table 9; experiment was not repeated for Ttr expression; Aqp1 expression analysis was repeated in one independent experiment). (f) Western blot analysis of Aqp1 and Otx2 expression in CP tumours and wild-type (WT) CPs at P14. β-actin serves as the loading control. Molecular size markers and representative unprocessed original scans of blots can be found in Supplementary Fig. 9.
Enhanced proliferation in Notch-induced CP tumour

Although CP epithelial cells in wild-type and Math1-Cre;Rosa26-EYFP mice (EYFP+/EYFP−) remain post-mitotic after birth, ~40% of NICD1+/GFP+ cells in Mcre;NICD1 mice are Ki-67+. This percentage gradually decreases to ~1% after 3 weeks of age (Fig. 2a,b and Supplementary Fig. 3a). EdU (5-ethynyl-2'-deoxyuridine) incorporation assays also revealed enhanced tumour cell proliferation in Mcre;NICD1 mice (Supplementary Fig. 4a). As tumour cells exit the cell cycle, Ccnd1 expression is downregulated, whereas Cdkn1b (p27 Kip1) expression is upregulated (Fig. 2c and Supplementary Fig. 4b). Cleaved caspase-3 expression is not detected (Supplementary Fig. 4d). Together, these results indicate that Notch-induced CP tumour undergoes enhanced proliferation transiently after birth.

Tumour cells express the CP marker Lmx1a, but not the mesenchymal marker MaFb (ref. 25), and the expression of Aqp1, transthyretin (Ttr), cytokeratins and Otx2 is consistently reduced compared with CP epithelial cells26 (Fig. 2d–f and Supplementary Figs 3b and 4e,f), indicating that sustained Notch signalling interferes with differentiation of tumour cells even after they become post-mitotic.

Abnormal Shh signalling in CP tumour cells

To identify signals that drive tumour cell proliferation, we compared transcriptional profiles of tumours and wild-type CPs at P0 (tumour cells are proliferative) and P21 (tumour cells are post-mitotic) using RNA-seq. Tumours and CPs clustered separately in principal component analysis, indicating distinct molecular profiles (Fig. 3a). Tumour cells exhibit gene expression profiles defined by differential expression of 2,738 (P0) and 4,964 (P21) transcripts (Supplementary Table 1 and Fig. 3b). Study of these differentially expressed transcripts identified 1,705 common targets, including Hes1 and Hes5, Aqp1, cytokeratins and Otx2, all of which show significant differential expression by quantitative PCR with reverse transcription (qRT–PCR) and immunostaining analyses, validating RNA-seq results (Figs 1g, 2d–f and 3e and Supplementary Figs 1c,d, 3b and 4e and Supplementary Table 1). Although tumour cells express higher messenger RNA levels for roof plate markers Lmx1a, Gdf7, Zic3, Zic4 and Msx2, the expression of many genes found in CP epithelium is significantly lower25–32 (Fig. 3c,e and Supplementary Table 1).

Comparison of tumour expression profiles at P0 and P21 uncovered 4,910 differentially expressed transcripts (Fig. 3b and Supplementary Table 2). We reasoned that differential genes unique to P0 tumour cells may include those involved in proliferation. To identify these genes, we excluded the 1,705 common differential targets between tumours and CPs at each time point to obtain 1,033 differential transcripts in tumours at P0 (Fig. 3b). We overlapped these 1,033 unique targets with the 4,910 differential genes between tumours of P0 and P21, further narrowing it down to 663 genes (Fig. 3b and Supplementary Table 3). Interrogating this shortened list of genes using ingenuity pathway analysis led us to a promising candidate: Shh signalling, which is also identified in analysis of the larger data set (Supplementary Tables 1 and 3). Tumour cells exhibit increased expression of Gli1, Gli2, Mycn and Ccnd1 compared with wild-type CP epithelium at P0 and P7. After P7, when tumour cells start to exit the cell cycle, the expression of these genes decreases to levels of those in control CPs, suggesting that decreased proliferation correlates with attenuated Shh signalling (Fig. 3d–f,i and Supplementary Fig. 4b).

In addition, the expression of p27 and Cdkn2b is upregulated in non-proliferating tumour cells, whereas Cdkn1c (p57 Kip2) is expressed at higher levels in mature CP (Fig. 2c and Supplementary Fig. 4c and Supplementary Table 1). Among hedgehog ligands, only Shh is abundantly expressed in hindbrain CP epithelial cells at birth and declines to undetectable levels after P14, resulting in lower expression levels in tumours than in wild-type CPs at P0 (Fig. 3d,e,g,i).

Shh drives CP tumour cell proliferation

To determine the role of Shh signalling in Notch-induced CP tumours, we treated tumour cells with a recombinant amino-terminal fragment of Shh (ShhN). Tumour cells formed spheres under serum-free conditions and continued to express Lmx1a, indicating intact lineage characteristics under these conditions (Fig. 4a,c). After 96 h, more Ki-67+ cells were detected in tumour spheres treated with ShhN than in untreated tumour spheres (Fig. 4b). Although epithelial cells formed aggregates that remained unresponsive, the size of tumour spheres in untreated tumours at P21 or later, whereas the Smo inhibitor cyclopamine abolished such effects, indicating that ShhN stimulates tumour cell proliferation (Fig. 4c,d). To determine whether tumour growth requires Shh, we treated Lcre;NICD1 and Mcre;NICD1 animals with the Smo inhibitor vismodegib (100 mg·kg−1) or vehicle daily from embryonic day 17.5 (E17.5) to P7, or from day E15.5 for 4 days, respectively33,34. Vismodegib treatment shrank hindbrain CP tumours, significantly reduced the number of tumour cells, and improved the survival of Lcre;NICD1 mice (Fig. 4e,f). Vismodegib also decreased tumour cell proliferation and suppressed Mycn expression in Mcre;NICD1 animals without affecting CP development in wild-type littermates (Fig. 4g–i and Supplementary Fig. 5). Together, these results indicate that Shh drives the growth of Notch-induced CP tumours.
**SHH and NOTCH signalling in human CP tumours**

We examined NOTCH and SHH signalling in human CP tumours. First, we used published data sets and analysed tumour transcriptomes, epigenomes and genomes. Principal component analysis of human CPPs and normal CPs revealed distinct molecular profiles for CPPs (ref. 13; Fig. 4j). MetaCore enrichment analysis of differential
Figure 4 Shh drives the proliferation of Notch-induced CP tumours. (a,b) Lmx1a (a, red) and Ki-67 (b, red) expression is shown in cultured tumour cells from Mcre;NICD1 mice. GFP (green) marks tumour cells, whereas DAPI staining (blue) labels nuclei. Scale bars, 10 μm. (c) Images of tumour cells and wild-type (WT) epithelial cells. Scale bar, 50 μm. (d) Quantification of tumour cells after 96-h treatments (n = 3 specimens per treatment per time point, raw data are available in Supplementary Table 9; mean ± s.e.m., two-way ANOVA, ****P < 0.0001; NS, not significant). (e) Kaplan–Meier curve depicting the survival of Lcre;NICD1 mice treated with vismodegib (n = 22 animals) or vehicle (n = 19 animals). (f) Hindbrain CPs from day P7 Lcre;NICD1 mice treated with vismodegib (lower) or vehicle (upper). Scale bar, 1 mm. (g) H&E staining of tumours from treated Mcre;NICD1 mice. Ki-67 expression (red) labels proliferating cells and GFP (green) marks tumour cells. DAPI staining (blue) labels nuclei. Scale bar, 25 μm. (h) Analysis of tumour cell proliferation in animals shown in g (n = 10 animals per treatment; mean ± s.e.m., two-tailed unpaired t-test, ***P < 0.001). (i) In situ hybridization analysis of Mycn expression in tumour (arrows) or epithelial cells (arrowheads) in animals shown in g. Scale bar, 25 μm. (j) Principal component analysis of human CPs (red dots; n = 7 tumours from seven individuals) and normal CPs (blue dots, n = 8 CPs from eight individuals). PC1 (horizontal): 37%, PC2 (vertical): 9.84%, PC3 (third dimension): 9.27%. (k) MetaCore analysis of differential genes in human CPs. Significantly enriched signalling networks (vertical): 9.84%, PC3 (third dimension): 9.27%. (l) Images of tumour cells derived from a single experiment, available in Supplementary Table 9 and overlapping genes between mouse and human CPs shown in i. (m) MetaCore analysis of overlapping genes between mouse and human CPs shown in l. (n) qRT–PCR analysis of gene expression in human CP tumours (CYP: n = 7 tumours from seven individuals; CPC: n = 2 tumours from two individuals). Values represent fold changes relative to human CP epithelium. Data shown are derived from a single experiment, available in Supplementary Table 9 and not repeated.
Figure 5 Notch-induced CP tumours possess a solitary primary cilium. (a) Transmission electron micrographs of primary cilia in tumour cells (arrow) from Mcre;NICD1 animals and wild-type (WT) CP epithelium (arrowheads). Scale bar, 0.5 μm. (b) The expression of cilia markers Arl13b (red) and γ-tubulin (red) in tumour cells is shown. Dotted lines mark the boundary between GFP+ (green) tumour cells and Aqp1+ (yellow) epithelium. DAPI staining (blue) labels nuclei. Primary cilia in epithelial (arrowheads, upper) or tumour cells (arrows, lower) are magnified in inset pictures. Scale bar, 10 μm. (c) The expression of Arl13b (red), γ-tubulin (red), and acetylated α-tubulin (act-α-tubulin, red) is shown in cultured CP cells. GFP (green) labels tumour cells and Aqp1 (yellow) marks epithelial cells. DAPI staining (blue) labels nuclei. Scale bar, 5 μm. (d) Hierarchical clustering of tumours and normal CPs at P0 based on 147 genes involved in cilia differentiation (n=3 specimens per genotype; one-way ANOVA, FDR < 0.05, fold change is shown). (e) qRT-PCR analysis of the expression of Mcidas and Foxj1 in tumours (black circles) and CPs (white circles) at different time points (data from technical replicates of each specimen in a single experiment are shown and available in Supplementary Table 9; experiment was not repeated for Mcidas expression; Foxj1 expression analysis was repeated in one independent experiment). (f) In situ hybridization analysis of the expression of Hes1 and Hes5 is shown in hindbrain roof plate (red dotted lines) and tumour cells (arrows) in Mcre;NICD1 or wild-type animals at embryonic day (E) 14.5. Scale bar, 100 μm. (g) Primary cilia are shown for cells in hindbrain roof plate (RP), CP epithelium, and tumours shown in f. Arl13b (red) and γ-tubulin (red) expression marks primary cilia and the basal body, respectively. DAPI staining (blue) labels nuclei. Scale bar, 10 μm. (h) Representative images show ARL13B expression (red) in human CP tumour cells (arrows) or normal CP epithelium (arrowhead). Primary cilia are magnified in inset pictures. DAPI staining (blue) labels nuclei. Scale bar, 10 μm. (i) Summary of cilia pattern in human CPPs (n=17 tumours from 16 individuals), CPCs (n=13 tumours from 13 individuals), and normal CPs (n=6 CPs from 6 disease-free individuals).
in CPPs compared with CPCs (refs 14,15; Supplementary Fig. 6a). MetaCore gene expression analysis showed significant enrichment for SHH and NOTCH pathways in human CPCs, an observation also supported by analysis of methylation and copy number variation data (Supplementary Fig. 6b–d and Supplementary Table 6). qRT–PCR analysis of human CP tumours revealed increased expression for genes of both pathways in CP tumours compared with normal human CP epithelial cells (Fig. 4n).

Expression profiling showed that many genes involved in ciliogenesis from a single experiment are shown, raw data are available in Supplementary Table 6. MetaCore gene expression analysis showed significant enrichment of tumour cells (ShhN: n = 3, 71.78 ± 6.93%, P < 0.0001; SAG: n = 3, 74.99 ± 3.03%, P < 0.0001; two-way ANOVA; Fig. 6c,d), even though both cell types express similar levels of Pcdh1 and Smo (Fig. 6e), indicating that Shh signalling in the primary cilium is preserved in tumour cells, but lost in epithelial cells despite their multiple primary cilia. Indeed, ShhN or SAG restored the percentage of Ki-67+ tumour cells after serum removal, an effect that can be reversed by cyclopamine, indicating that tumour cells are uniquely capable of responding to Shh through proliferation (Fig. 6f–h).

**NOTCH-induced CP tumour cells are monociliated**

In vertebrates, Shh-driven signalling occurs through the primary cilium16,17. To understand the mechanisms by which tumour cells with sustained Notch signalling respond to Shh, we examined primary cilia in CP tumour and epithelial cells. Transmission electron microscopy and staining for the cilia markers ADP-ribosylation factor-like 13b (Arf13b; ref. 35), γ-tubulin and acetylated α-tubulin revealed multiple short primary cilia in wild-type and EYFP+ epithelial cells in Math1–Cre;Rosa26-EYFP mice, whereas a single, longer primary cilium is present in tumour cells (epithelial cells: 1.70 ± 0.11 µm, n = 10; tumour cells: 3.78 ± 0.13 µm, n = 12; two-tailed unpaired t-test, P < 0.0001; data from a single experiment are shown, raw data are available in Supplementary Table 9; Fig. 5a–c and Supplementary Fig. 7a,b). Gene expression profiling showed that many genes involved in ciliogenesis are downregulated in tumour cells36–38 (Fig. 5d). The expression of forkhead box J1 (Foxj1) and multilicate differentiation and DNA synthesis associated cell cycle protein (Mccidas), two crucial regulators of the differentiation of cells with numerous motile cilia39–41, is consistently reduced in tumour cells (Fig. 5e and Supplementary Fig. 7c), indicating that Notch signalling suppresses Mccidas and Foxj1 expression, and blocks multilicate differentiation of tumour cells. Similar to tumour cells, progenitors in hindbrain roof plate exhibit increased expression of Hes1 and Hes5 at day E14.5, and possess single primary cilium (Fig. 5f,g and Supplementary Fig. 7d,e), suggesting that active Notch signalling preserves the single primary cilium of the progenitors during development. We characterized cilia pattern in human CP tumours (17 CPPs and 13 CPGs). Compared with normal human CP epithelial cells with multiple cilia, most CPPs comprise either monociliated tumour cells alone or mixed populations of monociliated and multiciliated cells. In all CPPs examined, cilia observed in tumour cells were solitary primary cilia (Fig. 5h,i and Supplementary Fig. 7f).

**Monociliated CP tumour cells are uniquely capable of transducing Shh signals**

To determine whether the distinct cilia pattern of tumour cells affects Shh signalling, we characterized Shh-driven Smo ciliary translocation. Both CP epithelial and tumour cells express Lmx1a when grown with serum; however, only tumour cells can proliferate (Fig. 6a,b and Supplementary Fig. 7g). After serum removal, cells were treated with ShhN or SAG, a Shh pathway agonist42 (Fig. 6a). Although such treatment failed to promote ciliary accumulation of Smo in epithelial cells, it led to translocation of Smo into the solitary primary cilium of tumour cells (ShhN: n = 3, 71.78 ± 6.93%, P < 0.0001; SAG: n = 3, 74.99 ± 3.03%, P < 0.0001; two-way ANOVA; Fig. 6c,d), even though both cell types express similar levels of Pch1 and Smo (Fig. 6e), indicating that Shh signalling in the primary cilium is preserved in tumour cells, but lost in epithelial cells despite their multiple primary cilia. Indeed, ShhN or SAG restored the percentage of Ki-67+ tumour cells after serum removal, an effect that can be reversed by cyclopamine, indicating that tumour cells are uniquely capable of responding to Shh through proliferation (Fig. 6f–h).

**Notch-induced CP tumour arises from roof plate progenitors**

The similarity in gene expression between tumour and roof plate cells, together with elevated Notch signalling and solitary primary cilium in the latter, suggests that Notch-induced CP tumour is related to the roof plate. To delineate the developmental origin of CP tumour, we first analysed the distribution of Atoh1+ progenitors in hindbrain roof plate using Math1+MIGFP mice with enhanced green fluorescent protein (EGFP) fused to the carboxy terminus of Atoh1 (ref. 43). In addition to the rhombic lip, Atoh1:EGFP+ cells are present in the Lmx1a+/Otx2+ upper roof plate but largely absent from the lower roof plate (Fig. 7a and Supplementary Fig. 8a). Second, we analysed tumour formation in Mcre;NIDC1 animals during development. At day E12.5, although many NIDC1+/GFP+ cells are located in the rhombic lip bordering the Lmx1a+ roof plate, some NIDC1+/GFP+ cells are present within CP forming into papillary structures (Fig. 7b). These prospective Lmx1a+ tumour cells undergo proliferation (Ki-67+), and remain undifferentiated (Aqp1+; Fig. 7b and Supplementary Fig. 8b). At day E14.5, most NIDC1+/GFP+ tumour cells are found in Lmx1a+ upper roof plate and the rostral half of the CP (Fig. 7c). Third, proliferative progenitors (Lmx1a+/Ki-67+/Aqp1+) in hindbrain roof plate differentiate into post-mitotic epithelial cells that form the CP epithelium (Lmx1a+/Ki-67+/Aqp1+) during embryogenesis44,45. The shape and size of the roof plate are similar between wild-type and Mcre;NIDC1 mice; however, tumour cells remain proliferative and undifferentiated (Ki-67+/Aqp1+) even after their incorporation into CP epithelium (Fig. 8a and Supplementary Fig. 8c). Together, these results indicate that CP tumours in these animals arise from roof plate progenitors and migrate into the CP where they continue to undergo Shh-driven proliferation throughout development (Fig. 8b,c).

**DISCUSSION**

In this study, we examined Notch signalling in CP tumours, a group of rare brain neoplasms most commonly found in children. Consistent with a role for NOTCH pathway in these tumours8–11, analysis of human CP tumour data sets revealed significant enrichment for NOTCH signalling. Using animal models, we showed that sustained Notch1 signalling leads to CP tumours that, similar to human CP tumours, express CP markers and undergo increased proliferation. Cross-species molecular analysis demonstrates that these CP tumours closely resemble their human counterpart, validating our animal models as accurate representations of human disease.

CP tumours are thought to originate from CP epithelium14,15. Analysis of Math1–Cre;Rosa26-EYFP mice indicates that EYFP+
Figure 6  Monociliated CP tumour cells are uniquely capable of transducing Shh signals. (a) Dissociated CP tumour cells from Mcre;NICD1 animals were initially cultured in the presence of 10% fetal bovine serum (FBS). Cultured CP tumour cells were then switched to serum-free conditions for 2 days followed by treatment with ShhN or SAG for 1.5 or 3 days for signalling and proliferation analysis, respectively. (b) The expression of Lmx1a (red) and Ki-67 (red) is shown in CP tumour cells cultured with FBS. GFP (green) labels NICD1 CP tumour cells, whereas Aqp1 marks CP epithelial cells. DAPI staining (blue) labels nuclei. Scale bar, 25μm. (c,d) After treatment with ShhN, SAG or control vehicle (Ctrl), Smo (red) localization is shown in CP tumour (c) or epithelial cells (d). Arl13b expression (green) marks primary cilia, whereas DAPI staining (blue) labels nuclei. Scale bar, 10μm. (e) qRT-PCR analysis of Ptc1 and Smo expression in CP tumours from Mcre;NICD1 animals (black circles) and wild-type CPs (WT, white circles) at P7 (data from technical replicates of each set of specimen in a single experiment are shown; experiment was not repeated; raw data can be found in Supplementary Table 9). (f) The expression of Ki-67 (green) in CP tumour cells from Mcre;NICD1 animals treated with ShhN for 48h is shown. Arl13b expression (red) labels primary cilia; DAPI staining (blue) marks nuclei. Note that Ki-67 expression is present in a monociliated CP tumour cell, whereas a multiciliated CP epithelial cell lacks Ki-67 expression. Scale bar, 10μm. (g) The expression of Ki-67 (red) in cultured CP tumour cells from Mcre;NICD1 animals which were treated with ShhN, SAG or control vehicle (Ctrl). GFP (green) labels NICD1 tumour cells, whereas DAPI staining (blue) marks nuclei. Scale bar, 25μm. (h) Quantification of the percentage of Ki-67+ cells in NICD1/GFP tumour cells after 72-h treatment as indicated (n = 4 specimens per treatment, data from a single experiment are shown, raw data are available in Supplementary Table 9; mean ± s.e.m., two-way ANOVA, ***P < 0.001; ****P < 0.0001).
Figure 7 Notch-induced CP tumours arise from progenitors in the roof plate. (a) Atoh1:EGFP expression in Math1<sup>M1GFP</sup> mice at day E14.5 is detected with a GFP antibody and shown as green signals. Lmx1a expression (red) in the roof plate (marked by dotted lines) is shown, DAPI staining (blue) labels nuclei. Note that Atoh1:EGFP<sup>+</sup> progenitors (arrows) are present only in the Lmx1a<sup>+</sup> upper roof plate. Scale bar, 50 μm. (b) Schematic illustration of hindbrain roof plate (RP) and CP at day E12.5. The solid line marks the transverse plane across the roof plate shown in the H&E staining image and diagram. The red outlined region in the diagram representing the interface of the rhombic lip and roof plate is shown in Mcre:NICD1 mice at day E12.5. GFP (green) marks Atoh1<sup>+</sup> rhombic lip progenitors and prospective tumour cells (arrows) in the roof plate and CP. The expression of Lmx1a (red) marks the roof plate/CP lineage. Ki-67 expression (red) labels proliferating cells, whereas Aqp1 expression (red) marks differentiated epithelial cells. DAPI staining (blue) labels nuclei. Scale bars, 1 mm (black) and 30 μm (white). (c) Gene expression in the roof plate and CP in Mcre:NICD1 mice at day E14.5. Outlined regions of progenitors within the upper (a1, a2) and lower (b1, b2) roof plate as well as tumour cells (c) in CP are shown at higher magnification. GFP expression (green) marks NICD1<sup>+</sup> tumour cells in the roof plate and CP. Lmx1a expression (red) labels the roof plate and CP lineage, whereas DAPI staining (blue) marks nuclei. Scale bar, 300 μm.

Epithelial cells derived from Atoh1<sup>+</sup> progenitors in the roof plate exhibit properties similar to the rest of the CP epithelium. Indeed, the identical CP tumours in Lcre:NICD1 and Mcre:NICD1 mice indicate that both Atoh1<sup>+</sup> and Atoh1<sup>-</sup> lineages in CP are sensitive to Notch signalling activation. However, despite the expression of CP markers, expression of many genes found in mature epithelium fails to be upregulated in tumour cells, suggesting a related but distinct developmental origin, or a block in differentiation, or both. The presence of Atoh1<sup>+</sup> progenitors and nascent tumour cells from Mcre:NICD1 mice in the roof plate suggests that CP tumours arise from progenitors within this region<sup>44,45</sup>. In agreement, tumour cells and these progenitors exhibit similar characteristics: increased expression of roof plate markers, elevated Notch signalling, and single primary cilium. The development of CP tumours induced by sustained Notch signalling may reflect its inherent role in roof plate/CP morphogenesis<sup>46-49</sup>: the Notch pathway suppresses multiciliate and epithelial differentiation of progenitors, thereby preserving the primary cilium-based Shh signalling (Fig. 8b). Compared with
Figure 8 Notch-induced CP tumour cells retain properties of roof plate progenitors. (a) Analysis of gene expression and cell proliferation in tumour cells and progenitors within hindbrain roof plate in Mcre;NICD1 and wild-type (WT) animals, respectively, at day E12.5. The expression of Lmx1a (red) marks the roof plate and CP lineage. Ki-67 expression (green) labels proliferating cells, and Aqp1 expression (green) marks differentiated epithelial cells. White dotted lines demarcate the domain of progenitors in hindbrain roof plate. Outlined regions of Lmx1a+/Ki-67−/Aqp1− progenitors (i, ii, v, vi), tumour cells (ii, iv) (arrows), and Lmx1a+/Ki-67+/Aqp1+/differentiated epithelial cells (ii, iv) (red dashed lines) are shown at higher magnification. Scale bars, 100 μm. (b) Schematic diagram of interaction between Notch and Shh pathways during the roof plate/CP morphogenesis and tumour formation. Progenitors in the roof plate (RP, orange) next to the rhombic lip (RL) exhibit active Notch signalling, possess a solitary primary cilium, and proliferate in response to Shh (green dots) secreted from multiciliated epithelial cells (grey). The self-renewal will cease as Notch pathway activity in progenitor cells is attenuated to allow for multiciliate differentiation, thereby abolishing the response of differentiating progenitors to Shh that drives their expansion during development. (c) Roof plate progenitors with constitutive Notch pathway activity (red) remain monociliated and undergo aberrant proliferation to become tumour cells that retain the ability to respond to Shh signals in the local environment and undergo Shh-driven proliferation.

CPCs, human CPPs express higher levels of roof plate markers (Supplementary Fig. 5a), consistent with their developmental origin.

We provide evidence that CP epithelial cells secrete Shh that drives the proliferation of a distinct population of Shh-responsive progenitors in the upper roof plate. Consistent with previous reports, we showed Shh expression in hindbrain CP epithelium at birth that gradually disappears within two weeks. Interestingly, low-level cell proliferation and Mycn expression is detected in tumour cells from Mcre;NICD1 animals at P14, presumably driven by residual or transvesicular Shh from other brain regions, as shown for CP-derived signals regulating distant cell populations in the brain. Tissue-specific deletion may delineate the role of Shh from these sources in tumour growth.

Analysis of published data sets demonstrated enrichment for SHH signalling, suggesting a role for deregulated SHH expression in human CP tumours, including CPCs (refs 13–15). Consistently, tumours from Mcre;NICD1 or Lcre;NICD1 mice exhibit active proliferation similar to that observed in CPCs at P0 when Shh is robustly expressed. Our results suggest that Shh pathway inhibition may represent a viable strategy for targeted therapy for CP tumours. SHH pathway inhibitors, clinically approved for treating other more common cancer types, may be ‘repurposed’ for treating patients with CP tumours exhibiting abnormal SHH signalling. Indeed, vismodegib treatment interferes with tumour progression in our models, although further validation with xenograft models would be necessary. The tumour becomes quiescent as Shh expression decreases, suggesting that additional changes are present to support aggressive growth of CP tumours as seen in human CPCs with aberrant NOTCH signalling. Tumour cells proliferate in the presence of serum (Fig. 6b and Supplementary Fig. 7g), indicating that serum may contain factors capable of driving tumour growth. Amplifications of TAF12, NFYC and RAD54L play an important role in CPCs (ref. 15). Their expression is not significantly changed in...
our CP tumours; however, deregulation of these genes may alter the behaviour of Notch-induced CP tumours. TP53 alterations play a crucial role in human CPCs (ref. 6,14). CP tumours in our models retain the wild-type Tp53 and exhibit signs of Tp53 signalling and DNA damage response (Supplementary Tables 1–3 and 5 and Fig. 4m), suggesting that Tp53 loss may facilitate malignant transformation of Notch-induced CP tumours.

Aberrant NOTCH signalling has been linked to different cancers in humans and therapeutically targeted55–57. The single primary cilium on tumour cells with constitutive Notch signalling proves to be essential for Shh signalling, whereas multiple primary cilia may interfere with cilium-dependent signalling activities46–51. A Notch-regulated signalling cascade involving Mcidas, Myb and FoxJ1 plays an important role in the differentiation of cells with multiple motile cilia40,41,42,64. Our results reveal that the interaction between Notch signalling and primary cilia is crucial for CP tumorigenesis (Fig. 8c): sustained Notch signalling preserves the single primary cilium by suppressing Mcidas and FoxJ1 expression to block multiciliate differentiation, transforming progenitors into tumour cells that undergo Shh-driven proliferation. Targeting Notch-mediated multiciliate differentiation may represent a rational strategy for CP tumour treatment. Human CP epithelial cells exhibit a ‘hobnail’ contour on the apical side, whereas the surface of CP tumour cells is more flattened65,66. Our results suggest that the cilia pattern of tumour cells may mediate this distinct morphology. Indeed, most human CP tumours (including all CPCs) exhibit solitary primary cilium. Understanding the interaction between Notch signalling, ciogenesis and epithelial differentiation in CP tumours is essential to validate the therapeutic potential of Notch inhibitors.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Note:** Supplementary Information is available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

L.I. and H.Z. conceived and planned the project, and wrote the manuscript. H.C.G.W. and M.L.C. reviewed diagnoses of human tissue samples. J.W., J.O., R.J.W.-R. and U.S. analysed morphological characters and gene expression patterns of human tumour samples. M.P.L. and M.K.L. performed cilia and gene expression analyses in human tissue samples. K.B.G. provided assistance with gene expression analysis. E.Z. conducted RNA-seq data processing and analysis. J.L.S. provided technical advice, support and data analysis for electron microscopy studies.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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Multiple sets of CP specimens from Table 9 for information on animals used for each experiment. Dissected CP specimens from the same genotype. Gender information is not available for animals at P0 and P7. Mcre;NICD1 mice, reviewed by two neuropathologists (H.G.W., L., S. Santagata) using standard Diagnoses of human CP specimens from Boston Children’s Hospital were normal human CP epithelial cells (ScienCell) were used at Sanford Research, Sanford Burnham Prebys Medical Discovery Institute, Human CP specimens were procured with informed consent from human subjects following the requirements by institutional review boards at Sanford Research, Sanford Burnham Prebys Medical Discovery Institute, and Ludwig-Maximilians-University. All CP specimens from Boston Children’s Hospital were obtained under an approved institutional review board protocol (Supplementary Table 7). Normal human CP epithelial cells (ScienCell) were used as the control in gene expression analysis. All tissues were handled in accordance with guidelines and regulations for the research use of human brain tissue set forth by the NIH (http://osp.od.nih.gov/office-clinical-research-and-bioethics-policy). Diagnoses of human CP specimens from Boston Children’s Hospital were reviewed by two neuropathologists (H.G.W., S. Santagata) using standard WHO criteria.

Isolation and culture of primary CP cells. Multiple sets of CP specimens from Mcre;NICD1 and/or wild-type mice were collected. To obtain sufficient numbers of cells, each set of specimens included tissues pooled from multiple animals of the same genotype. Gender information is not available for animals at P0 and P7. Both male and female animals were used at other time points (see Supplementary Table 9 for information on animals used for each experiment). Dissected CP specimens were dissociated with forces under a stereoscope followed by enzymatic digestion at 37 °C for 20 min in 0.7 mg/ml−1 of hyaluronidase (H3506, Sigma-Aldrich), 0.2 mg/ml−1 of kynurenic acid (Sigma-Aldrich K3375), and 1 mg ml−1 of trypsin in Hank’s balanced salt solution (HBSS, 14170-112; Life Technologies) supplemented with 2 mM glucose. The trypsin inhibitor ovomucoid (LS003085, Worthington Biochemical Corporation) was added to stop enzymatic digestion and dissociated CP cells were centrifuged at 200g for 5 min at 4 °C. Cell pellets were resuspended in Dulbecco’s modified Eagle’s medium/Nutrient Mixture F-12 Ham’s Liquid Media (DMEFM/F12, SH30271; Thermo Fisher Scientific), and cultured in DMEM/F12 supplemented with 30 ng ml−1 of EGF (Sigma-Aldrich E4127), 30 ng ml−1 of FGFl (Sigma-Aldrich F0291), B27 supplement, 2 mM glutamine, and 100 µM penicillin/streptomycin (all from Life Technologies). After treatment under serum-free conditions for 96 h, cells were dissociated mechanically by pipetting and quantified. For culture with serum, medium was supplemented with 10% fetal bovine serum. Cytosine β-arabinofuranoside (ara-C, 20µM; Sigma-Aldrich C1768) was added for the following day to eliminate contaminating fibroblasts. Cultured cells were treated with ShHH (ref. 67; 200 ng ml−1), or SAG (200 mM; 11914, Cayman Chemical Company), with or without cyclopamine (10µM; LC laboratories C-8700). Primary CP tumour or epithelial cells were not listed in the database of commonly misidentified cell lines maintained by JCLAC and NCBI BioSample. Analyses of gene expression, proliferation and signal transduction were performed in cultured primary CP cells (Figs 4a–d and 6b–d,f–h and Supplementary Fig. 7g). Results from these studies confirmed their identity. Given the short time (~8 days) during which CP cells were maintained as primary cultures supplemented with antibiotics, we did not test for mycoplasma contamination.

Histology, immunohistochemistry, immunofluorescence and immunocytochemistry. Whole brains were fixed overnight in 4% paraformaldehyde (PFA) and processed for paraffin embedding and sectioning. Tissue sections were deparaffinized with CitriSolv (Decon Labs), and then rehydrated with graded ethanol series. For frozen tissues, samples were further equilibrated in 20% sucrose at 4 °C for 24–48 h, embedded in Tissue-Tek Optimal Cutting Temperature (O.C.T.) compound (Sakura Finetek), and sectioned (15–20 µm thickness) on a cryostat. Cultured cells were fixed in 4% PFA at room temperature for 10 min and stored in phosphate-buffered saline (PBS). For primary cilia staining, cells were fixed with cold methanol for 10 min and stored in PBS at 4 °C.

No randomization was used to determine how samples were allocated to experimental groups and processed. Immunostaining was carried out as described previously. Heat-induced epitope retrieval was performed for paraffin-embedded tissue sections in Rodent Decloaker (Biocare Medical). For immunohistochemistry, controls were performed by pre-treating with non-labeled antibodies and fluorescently labelled secondary antibodies (Jackson ImmunoResearch). Primary antibodies used and dilution ratios are: mouse monoclonal anti-acetylated α-tubulin (1:500, ab24610, clone 6-11B-1, abcam), mouse monoclonal anti-acetylated α-tubulin (1:500, Sigma-Aldrich T7451, clone 6-11B-1), mouse monoclonal anti-Aurh13b (1:500, clone N29586, NeuroMab), rabbit anti-Aurh13b (1:500, 17711-1-AP, Proteintech), mouse monoclonal anti-aquaporin 1 (1:1,000, clone 1/22, abcam ab9566), rabbit anti-aquaporin 1 (1:1,000, AR2219, EMD Millipore), rabbit monoclonal anti-cleaved caspase-3 (Asp175; clone 316E1, 1:800, 9664, Cell Signaling Technology), mouse monoclonal anti-Cdcn1 (1:100, sc-450, clone 72-13G, Santa Cruz Biotechnology), mouse monoclonal anti-Cdkn1b (p27 Kip1; 1:100, 610242, clone 57/Kip1/p27, BD Biosciences), rabbit anti-cytokeratins (1:100, 20622, Dako, Carpinteria), mouse monoclonal anti-FosJ1 (1:50, 14,9965, Clone 2A5, EbiScience), chicken anti-GFP (1:1,000, G0610, Aviva Systems Biology), mouse monoclonal anti-γ-tubulin (Sigma-Aldrich T6557, clone GTU-88, 1:1,000), guinea pig anti-Heis1 (1:150, from R. Kageyama), rabbit monoclonal anti-Ki-67 (abcam ab16667, clone SP6, 1:100), goat anti-Lmx1a (Santa Cruz sc-54273, 1:1000), rabbit anti-MafB (1:200, IHC-00351, Bethyl Laboratories), rabbit anti-Otx2 (EMD Millipore AB9566, 1:500), rabbit anti-Smo (ref. 6; 1:100, sheep anti-transthyrein (abcam ab9015, 1:500).

The investigator was blinded to group allocation in the following two experiments. First, EYFP+ or GFP+ cells in 300 Ots2+ cells were assessed by analysing three distinct tissue regions of each sample. The percentage of EYFP+ or GFP+ cells was obtained by averaging the numbers of EYFP+ or GFP+ cells per 100 Ots2+ cells of all samples for each genotype at each time point. Second, in analysis of cell proliferation, the numbers of Ki-67+ cells in 300 Lmx1a+ or GFP+ cells were assessed from three distinct tissue regions for each animal. The percentage of Ki-67+ cells in the total Lmx1a+ or GFP+ cell population was calculated by averaging the numbers of Ki-67+ cells per 100 Lmx1a+ or 100 GFP+ cells of all samples for each genotype at each time point or each treatment. For proliferation analysis of cultured cells, Ki-67 expression was assessed by analysing three distinct fields: the percentage of Ki-67+ cells was calculated by averaging the numbers of Ki-67+ cells per 100 cells of all samples for each treatment.

For primary cilia staining of CP specimens from mice, the cilia pattern was assessed by analysing three distinct tissue regions of each animal. To measure the length of primary cilia, 12 primary cilia from tumour cells and 10 primary cilia from epithelial cells were examined. Smo ciliary translocation was assayed by analysing three distinct fields: the percentage of cells with ciliary accumulation of Smo was calculated by averaging the number of cells with Smo translocation per 100 cells of all samples for each treatment. Information on animals used for each experiment can be found in Supplementary Table 7.

For staining of primary cilia in human CP tumours and normal human CPs, the investigator was blinded to group allocation. Tissues used include: normal CP: 6 disease-free individuals; CPP: 17 tumour specimens from 16 individuals; CPC: 13 tumours from 10 individuals (Supplementary Table 9). Cilia staining was assessed by analysing 5 distinct tissue regions.

EdU (5-ethyl-2′-deoxyuridine) incorporation assay. One hour after intraperitoneal injection of 100 mg·kg−1 EdU (7180, Setareh Biotech), whole brains were collected from animals. Tissue sections were rinsed with Tris-buffered saline (TBS) and then incubated for 20 min with a solution containing 100 mM pH 8.5 Tris, 1 mM CuSO4, 10 mM Cy5-azide (B3030, Lumiprobe) and 100 mM ascorbic acid. Information on animals used is provided in Supplementary Table 9.

Image acquisition. Whole-mount images were obtained using a Nikon SMZ1000 stereomicroscope. Light and fluorescent microscopic images were obtained by a Nikon Eclipse 90i microscope system or a Nikon confocal microscope system A1+

Transmission electron microscopy. CP specimens were collected at different time points. To ensure collection of a sufficient amount of normal CP specimens, each normal CP specimen includes tissues pooled from two wild-type animals for P7, P14 and P22. Information on animals used is available in Supplementary Table 9. The investigator was blinded to group allocation. Tissues were fixed in 4% PFA, 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4 °C overnight. After washing
with cacodylate buffer supplemented with 10% sucrose, tissues were post-fixed with 1% osmium tetroxide (OsO₄), followed by incubation with 1% uranyl acetate in 30% ethanol. Tissue samples were dehydrated, then transferred to propylene oxide, and embedded in Eponate-12 epoxy resin (Ted Pella). Tissue samples were sectioned (85 nm) using a Leica UC-6 ultramicrotome. Sections were then stained with uranyl acetate and lead citrate, and observed under the JEOL 1400 transmission electron microscope (JEOL). Images were taken with a Gatan UltraScan 1000 CCD (charge-coupled device) digital camera (Gatan).

Immunoblotting. Immunoblotting was carried out as described previously. Multiple sets of CP specimens were collected at each time point. To obtain a sufficient quantity of protein, each set of specimens may contain tissues pooled from several animals of the same genotype. Gender information is not available for animals at P0 and P7 (see Supplementary Table 9 for information on animals used). Tissues were homogenized in protein lysis buffer (Tris/HCl pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% NP40, 0.25% sodium deoxycholate, 1x protease inhibitor cocktails (Research Product International)). Protein concentrations were measured by a standard BCA assay (Life Technologies). Equal amounts of protein were resolved in a reduced sample buffer, separated by SDS-PAGE, and then transferred to a polyvinylidene difluoride membrane. After incubation with 5% non-fat milk in TBST (TBS, 0.5% Tween 20) for 1 h, the membrane was incubated with primary antibodies for 1 h. The membrane was washed and incubated with horse radish peroxidase (HRP)-conjugated secondary antibodies (GE Healthcare) for 1 h. After washes, chemiluminescence detection was performed using Western Lightning Plus-ECL (PerkinElmer). β-actin was used as a protein loading control. Primary antibodies used include: mouse monoclonal anti-β-actin (Sigma-Aldrich A5441, clone AC-15, 1:2,000), rabbit anti-aquaporin 1 (EMD Millipore AB219, 1:1,000), mouse monoclonal anti-Cnd1 (Santa Cruz sc-450, clone 72-13G, 1:200), rabbit anti-Hes1 (R. Kageyama, 1:1,000), rabbit anti-Orx2 (EMD Millipore AB9566, 1:500).

RNA preparation, quantitative RT-PCR, in situ hybridization, microarray, and RNA-seq. For murine tissues, three independent sets of tumour specimens from Mcre;NICD1 mice and three sets of CP specimens from wild-type mice were collected at each time point. To ensure that a sufficient amount of RNA can be extracted, each set of specimens included tissues pooled from several animals of the same genotype. Gender information was not available for animals at P0 and P7 (see Supplementary Table 9 for information on animals used for each set of specimens). For human tissues, seven CPPs from seven individuals, two CPCs from two individuals, and normal human CP epithelial cells (ScienCell) were used (Supplementary Table 8). Total RNA was extracted using Trizol and PureLink RNA Mini Kit (Life Technologies) according to the manufacturer's instructions. For quantitative PCR with reverse transcription (qRT-PCR), total RNA samples were converted to cDNA using the GoScript Reverse Transcription System (Promega). All reactions were set up in triplicate with Absolute Blue QPCR Mix (Thermo Fisher Scientific) and run on an ABI 7900 Sequence Detection System (Life Technologies). Gene-specific primers and probes for mouse Agpl, Trr, Gli1, Mycn, Shh, Ptc1h, Smo, Hes1, Hes5, Fosj1, Meidas, and control Actnb were used (Supplementary Table 8). The following Taqman assays for human genes were used (Life Technologies): GJA1 (Hs01107666_m1), PTCH1 (Hs00181117_m1), MYCN (Hs00232074_m1), HES5 (Hs00378438_g1), HES1 (Hs01728787_m1), HEY1 (Hs0114113_m1), ACTB (Hs00607939_m1). Data were analysed using ABI Fast System SDS software. Transcript levels were determined as the number of transcripts for each gene relative to those of Actb (mouse) or ACTB (human). The results for each set of specimens were obtained by averaging transcript levels of technical triplicates and used for subsequent analyses. Data from qRT-PCR experiments can be found in Supplementary Table 9. Exclusion was applied only on rare occasions when one of the three wells of the triplicate was a significant outlier; however, for each sample in question, qRT-PCR experiments were repeated in independent experiments, which validated the exclusion.

In situ hybridization was performed by RNA In Situ Hybridization Core facility at Baylor College of Medicine as described previously. For analysis of Shh, Mycn and Gli1 expression, CP specimens from multiple animals were collected at P0 and P14. For analysis of Mycn expression in Mcre;NICD1 animals treated with vismodegib or vehicle, three tumours from three vismodegib-treated mice and five tumours from five vehicle-treated mice were examined at P0. For Hes1 and Hes5 expression analysis, two Mcre;NICD1 mice and one wild-type mouse were collected at day E14.5. Gender information was not available for embryos. Tissues were post-fixed with 10% sucrose, tissues were dehydrated and embedded in Eponate-12 epoxy resin (Ted Pella). Tissue samples were sectioned (85 nm) using a Leica UC-6 ultramicrotome. Sections were stained with uranyl acetate and lead citrate, and observed under the JEOL 1400 transmission electron microscope (JEOL). Images were taken with a Gatan UltraScan 1000 CCD (charge-coupled device) digital camera (Gatan).

For RNA-seq experiments, total RNA samples were ribo-depleted using the Ribominus Eukaryote System (Life Technologies), and used to generate sequencing libraries of barcoded fragment using the Ion Total RNA-Seq Kit V2 (Life Technologies). Libraries were sequenced on the Ion Proton sequencer, three libraries per Ion Proton P1 Chip, using 200 bp sequencing reagents. Reads were aligned to the mouse genome (mm10) using a combination of Tophat2 and Bowtie2. Differentially expressed transcripts were detected using the Cufflinks RNA-seq package (http://cufflinks.cs.vu.nl) and transcripts with a q value <0.05 (Cuffdiff) were analysed by ingenuity pathway analysis (Ingenuity Systems). Principal component analysis was performed using R package rgl (http://cran.r-project.org/web/packages/rgl/index.html), a three-dimensional visualization system based on OpenGL. Hierarchical clustering was performed using Genesis (http://genome.tugraz.at/genesilicent/genesilicent_description.shtml).

Statistical analyses and reproducibility. Statistical analyses were performed with GraphPad Prism 6.0 (GraphPad Software). All pooled data were expressed as the mean ± s.e.m. Differences between two groups were compared using an unpaired two-tailed t-test. Differences between multiple groups were analysed with one-way or two-way ANOVA followed by Tukey's multiple comparisons test. Results were considered significant at P < 0.05 (**), P < 0.01 (***), P < 0.001 (***) or P < 0.0001 (** ** **). For western blotting, analyses of the expression levels of Agpt1 and Otx2 at P14, and Hes1 expression at P0 and P7, were repeated in one independent experiment. Analyses of the expression levels of Hes1 at P90, and Cndcd1 expression were repeated in two independent experiments with similar results. RNA-seq and microarray studies were performed in single experiments. For in situ hybridization, analyses of Mycn expression in vismodegib-treated animals, and Mycn and Gli1 expression at P0 and P14, were repeated in one independent experiment with similar results. Analyses of Shh, Hes1 and Hes5 expression was performed in single experiments (Supplementary Table 9). SHH expression analysis in human tissues was conducted in one experiment with samples divided into two groups. For qRT-PCR analysis, the expression of Hes1, Hes5, Trr, Ptc1h, Smo and Mecdas was examined in single experiments, whereas Agpl1, Gli1, Mycn, Shh and Fosj1 expression analysis was repeated in one independent experiment with similar results. qRT-PCR analysis of human expression samples was performed in a single experiment. Analyses of cell proliferation (Ki-67 or EdU staining), percentage of EYFP expressing cells in CP epithelium, tumour cell numbers, and Smo translocation were performed in single experiments. The measurement of cilia length was repeated in one independent experiment with similar results. Representative images were selected from two independent experiments.

Accession numbers. Published human CP tumour data sets (GSE41998, GSE60886) were downloaded from GEO database for analysis. Data sets from our studies were deposited at NCBI: Sequencing: BioProject ID, PRJNA282889; Microarray: GSE71723.

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**Supplementary Figure 1** Morphological and gene expression analysis of CPs in \textit{Mcre;NICD1} mice. (a) EYFP expression (green, arrowheads) detected with a GFP antibody demarcates CP epithelial cells derived from Atoh1\(^+\) progenitors in \textit{Mcre;EYFP} mice, while GFP expression (green) labels NICD1\(^+\) tumour cells in \textit{Mcre;NICD1} mice at P0, P7, P14, P21, and P90. The expression of Otx2 (red) marks CP epithelial cells. DAPI staining (blue) labels nuclei. Scale bar: 25 µm. (b) H&E staining of CPs from \textit{Mcre;NICD1} mice and wild type (WT) littermates at P0, P7, P14, and P21. Boxed regions of the abnormal growth of CP in the enlarged 4\(^{th}\) ventricle of \textit{Mcre;NICD1} animals are shown in higher magnification. Notice that wild type CP epithelium exhibits the “hobnail” configuration (arrowheads), while abnormal CP growth in \textit{Mcre;NICD1} mice displays a “flattened” appearance on ventricular surfaces (arrow). Scale bars: white, 250 µm; black: 40 µm. (c) The expression of Hes1 (red) in CPs from \textit{Mcre;NICD1} mice. GFP expression (green) labels NICD1\(^+\) CP cells. Dotted lines mark the boundary between CP epithelial cells and NICD1\(^+\) cells. DAPI staining (blue) labels nuclei. Scale bar: 25 µm. (d) Western blot analysis of Hes1 expression in CP tumours from \textit{Mcre;NICD1} mice and wild type (WT) CPs at P0, P7 and P90. β-actin serves as loading control. Molecular size marker and representative image of unprocessed blots can be found in Supplementary Figure 9.
Supplementary Figure 2  Morphological analysis of CPs in Lcre;NICD1 mice. (a) Bright field images of brain hemispheres in Lcre;NICD1 mice and wild type (WT) littermates at P14. Notice that Lcre;NICD1 mice develop abnormal growth of CP (dotted red lines) and display enlarged lateral ventricle (arrows). Vesicular sacs with accumulated cerebrospinal fluid are shown (asterisks). Scale bar: 1 mm. (b) H&E staining of CPs in hindbrain and lateral ventricles of Lcre;NICD1 animals and wild type littermates at P7, P14, and P30. Notice that compared to CPs in wild type (WT) littermates (arrowheads), Lcre;NICD1 mice develop abnormal CP growth (arrows) and exhibit enlarged 4th and lateral ventricles. Vesicular sacs with accumulated cerebrospinal fluid are shown (asterisks). Scale bars: white, 100 µm; black: 300 µm.
Supplementary Figure 3  Analysis of proliferation and gene expression in CP tumours from Lcre;NICD1 mice. (a) The expression of Ki-67 in tumour cells in the hindbrain and lateral ventricles of Lcre;NICD1 animals at P0, P7, P14, and P90, respectively. Dotted lines mark the boundary of tumours in lateral ventricles. Notice that the expression of Ki-67 in tumour cells is gradually reduced over time. Scale bar: 25 µm. (b) The expression of Lmx1a, Otx2, Aqp1, and cytokeratins in CP tumours in the hindbrain and lateral ventricles of Lcre;NICD1 mice at P7. CPs at these sites from P7 wild type (WT) littermates serve as control. Dotted lines mark the boundary of the 4th and lateral ventricles. Notice that tumour cells display reduced expression of Aqp1 and cytokeratins. Scale bar: 25 µm.
Supplementary Figure 4  Analysis of CP tumours from Mcre;NICD1 mice and CPs of wild type (WT) mice. (a) Results of 5-ethynyl-2'-deoxyuridine (EdU) incorporation assays are shown for tumours at P0, P7, and P14, respectively. EdU staining (magenta) labels cells in S-phase of the cell cycle. GFP expression (green) labels NICD1+ tumour cells. Dotted lines mark the boundary between GFP+ tumour cells and CP epithelium. DAPI staining (blue) labels nuclei. Scale bar: 25 µm. (b) The expression of cyclin D1 (Ccnd1) in tumours from Mcre;NICD1 mice at P0 and P14, respectively. Aqp1 expression (green) labels CP epithelium, while Ccnd1 expression (red) labels proliferating cells. Dotted lines mark the boundary between proliferative tumour cells and Aqp1+ epithelial cells. DAPI staining (blue) labels nuclei. Scale bar: 25 µm. (c) The presence of EdU-incorporating cells (magenta) and Cdkn1b+ (p27 Kip1, green) cells in tumours and WT at P7. Though tumours contain abundant cells positive for EdU or p27 expression, staining for EdU or p27 is mutually exclusive. Notice that p27+ or EdU-incorporating cells are absent in wild type CPs despite positive staining in neighboring cerebellum (Cb). Scale bar: 25 µm. (d) The expression of Cleaved Caspase-3 in CP tumours and WT CPs at P7. Cleaved Caspase-3 expression (red) is undetectable in tumour and CPs. DAPI staining (blue) marks nuclei. Scale bar: 25 µm. (e) Gene expression in WT CP epithelium at P7. The expression of Lmx1a (red), Otx2 (red), and Aqp1 (red) labels CP epithelial cells. DAPI staining (blue) marks nuclei. Scale bar: 25 µm. (f) The expression of mesenchyme marker MafB in CPs from Mcre;NICD1 animals at P7. GFP (green) labels NICD1+ tumour cells. Dotted lines mark the boundary between GFP+ tumour cells and CP epithelium. MafB expression (red) is detected in normal CP tissue, but absent from GFP+ tumour cells. DAPI staining (blue) marks nuclei. Scale bar: 25 µm.
Supplementary Figure 5 Morphological and gene expression analysis of CPs from treated wild type animals. H&E staining of CPs from wild type mice treated with either vismodegib or vehicle from day E15.5 for 4 days. The expression of Ki-67 (red) labels proliferating cells. Lmx1a expression (green) marks CP epithelial cells. DAPI staining (blue) labels nuclei. Notice that Ki-67+ proliferative cells are present in neighboring cerebellum (Cb); however, there is no Ki-67+ cells in CP epithelium. Scale bar: 25 µm.
Supplementary Figure 6 Transcriptome, epigenome, and genome analysis of human CP tumours. (a) Hierarchical clustering of human CPPs and CPCs based on the expression of roof plate genes (CPP: n=24 tumours from 24 individuals; CPC: n=15 tumours from 15 individuals; one-way ANOVA, FDR < 0.05, fold change is shown). (b) MetaCore gene enrichment analysis of differentially methylated genes between human CPPs and CPCs (CPP: n=5 tumours from 5 individuals; CPC: n=15 tumours from 15 individuals). (c) Schematic illustration of results from MetaCore network analysis of differentially methylated genes between human CPPs and CPCs shown in (b). The red colored nodes indicate relatively higher expression and green nodes indicate relatively lower expression in CPPs compared to CPCs. (d) MetaCore gene enrichment analysis of copy number variations in human CP tumours (CPP: n=37 tumours from 37 individuals; CPC: n=38 tumours from 38 individuals). (e) Representative images show in-situ hybridization analysis of SHH expression in normal human CP (n=5 CPs from 5 disease-free individuals) and human CP tumours (CPP: n=11 tumour specimens from 10 individuals; CPC: n=10 tumours from 10 individuals). Notice that SHH expression is present in normal hindbrain CP epithelium (arrowheads) and tumour tissue (arrows). Signal intensity varied among samples, likely as a result of brain regions examined, conditions under which specimens were collected and stored, and the length of time of specimens in storage. Representative images are shown. Scale bar: 50 µm.
Supplementary Figure 7 Analysis of primary cilia and gene expression. (a) Transmission electron micrograph of primary cilia on CP tumour cells (arrow) or CP epithelium (arrowheads). Scale bar: 0.5 µm. (b) The expression of Arl13b (red) and γ-tubulin (red) in EYFP+ CP epithelial cells of Mcre;EYFP mice. EYFP (green) detected with a GFP antibody demarcates epithelial cells derived from Atoh1+ progenitors in Mcre;EYFP mice, while Aqp1 (yellow) marks differentiated epithelial cells. DAPI staining (blue) labels nuclei. Inset pictures show high magnification images of EYFP+ (arrows, upper) or EYFP- epithelial cells (arrowheads, lower). Scale bar: 10 µm. (c) The expression of Foxj1 (red) in CP tumour cells from Mcre;NICD1 mice at P14. Dotted lines mark boundary between tumour cells and Aqp1+ (green) CP epithelium. DAPI staining (blue) labels nuclei. Notice that Foxj1 expression is reduced in tumour cells compared to epithelial cells. Scale bar: 25 µm. (d) Schematic illustration of hindbrain roof plate and CP in mouse embryo at day E14.5. Solid line marks the transverse plane across the cerebellum, roof plate, and CP. H&E staining and the diagram show structures in this plane. LRL: lower rhombic lip; URL: upper rhombic lip; LRP: lower roof plate; URP: upper roof plate; CB: cerebellum. Scale bar: 1 mm. (e) Cilia pattern in cells within the roof plate and CP epithelium of wild type animals is shown at day E14.5. Arl13b (red) and γ-tubulin expression (red) marks primary cilia and basal bodies, respectively. DAPI staining (blue) labels nuclei. Scale bar: 10 µm. (f) Representative images show the expression of acetylated α-tubulin (act-α-tubulin, red) and AQP1 (red) in normal human CP and human CP tumours. solitary cilium (arrows) or multiple cilia (arrowheads) are magnified in inset pictures. Hoechst staining (blue) labels nuclei. Scale bar: 10 µm. (g) The expression Lmx1a (red) and Ki-67 (red) in CP cells from Mcre;NICD1 mice cultured in the presence of serum. GFP (green) labels NICD1+ tumour cells, while Aqp1 marks epithelial cells. DAPI staining (blue) labels nuclei. Scale bar: 25 µm.
Supplementary Figure 8 Notch-induced CP tumours arise from progenitors in the roof plate. (a) Atoh1:EGFP expression in Math1M1GFP mice at day E14.5 is detected with a GFP antibody and shown in green signals. Otx2 expression (red) labels the roof plate (marked by dotted lines), while DAPI staining (blue) marks nuclei. Notice that Atoh1:EGFP+ progenitors (arrows) are present in Otx2+ upper roof plate, but absent in lower roof plate. Scale bar: 50 µm. (b) Gene expression in CPs from Mcre:NICD1 mice at day E14.5. GFP (green) marks tumour cells. Ki-67 expression (red) labels proliferating cells, and Aqp1 expression (red) marks differentiated epithelial cells. DAPI staining (blue) labels nuclei. Scale bar: 30 µm. (c) Analysis of gene expression and proliferation of tumour cells and progenitors within hindbrain roof plate in Mcre;NICD1 and wild type (WT) mice, respectively, at day E14.5. The expression of Lmx1a (red) marks the roof plate and CP lineage. While Ki-67 expression (green) labels proliferating cells, Aqp1 expression (green) marks differentiated epithelial cells. White dotted lines mark domain of progenitors (Lmx1a+/Ki-67+/Aqp1−) in hindbrain roof plate. Boxed regions in upper roof plate are shown in higher magnification. Red bracket lines mark differentiated epithelial cells (Lmx1a+/Ki-67+/Aqp1+). Stars demarcate tumour cells (Lmx1a+/Ki-67−/Aqp1−). Scale bar: 25 µm.
Supplementary Figure 9 Representative unprocessed scanned images of blots. Western blot analysis of Hes1 (a), Aqp1 and Otx2 (b), and Ccnd1 (c) expression in CP tumours from Mcre;NICD1 mice and CPs from wild type (WT) mice at different time points. β-actin serves as loading control. N/A: protein samples for unrelated study run on the same gel (c).
Supplementary Table Legends

Supplementary Table 1 Analysis of differentially expressed genes (FDR<0.05) between tumours and CPs at P0 and P21.

Supplementary Table 2 Analysis of differentially expressed genes (FDR<0.05) between tumours at P0 and P21.

Supplementary Table 3 Analysis of overlapping differentially expressed genes in tumours between P0 and P21 and between tumours and CPs at P0.

Supplementary Table 4 Analysis of gene expression in human CPPs.

Supplementary Table 5 Analysis of common differentially expressed genes in CPPs compared to normal CPs between human and mouse.

Supplementary Table 6 Analysis of gene expression in CPPs and CPCs in humans.

Supplementary Table 7 Case information for human tissues from Boston Children's Hospital.

Supplementary Table 8 Sequence of primers and probes for Q-RT-PCR.

Supplementary Table 9 Statistics source data and sample information.