Disruption of GMNC-MCIDAS multiciliogenesis program is critical in choroid plexus carcinoma development

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Multiciliated cells (MCCs) in the brain reside in the ependyma and the choroid plexus (CP) epithelia. The CP secretes cerebrospinal fluid that circulates within the ventricular system, driven by ependymal cilia movement. Tumors of the CP are rare primary brain neoplasms mostly found in children. CP tumors exist in three forms: CP papilloma (CPP), atypical CPP, and CP carcinoma (CPC). Though CPP and atypical CPP are generally benign and can be resolved by surgery, CPC is a particularly aggressive and little understood cancer with a poor survival rate and a tendency for recurrence and metastasis. In contrast to MCCs in the CP epithelia, CPCs in humans are characterized by solitary cilia, frequent TP53 mutations, and disturbances to multiciliogenesis program directed by the GMNC-MCIDAS transcriptional network. GMNC and MCIDAS are early transcriptional regulators of MCC fate differentiation in diverse tissues. Consistently, components of the GMNC-MCIDAS transcriptional program are expressed during CP development and required for multiciliation in the CP, while CPC driven by deletion of Trp53 and Rb1 in mice exhibits multiciliation defects consequent to deficiencies in the GMNC-MCIDAS program. Previous studies revealed that abnormal NOTCH pathway activation leads to CPP. Here we show that combined defects in NOTCH and Sonic Hedgehog signaling in mice generates tumors that are similar to CPC in humans. NOTCH-driven CP tumors are monociliated, and disruption of the NOTCH complex restores multiciliation and decreases tumor growth. NOTCH suppresses multiciliation in tumor cells by inhibiting the expression of GMNC and MCIDAS, while Gmnc-Mcidas overexpression rescues multiciliation defects and suppresses tumor cell proliferation. Taken together, these findings indicate that reactivation of the GMNC-MCIDAS multiciliogenesis program is critical for inhibiting tumorigenesis in the CP, and it may have therapeutic implications for the treatment of CPC.

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INTRODUCTION

The choroid plexus (CP) in each brain ventricle consists of stromal vasculatures ensheathed by epithelia [1–3]. The CP is responsible for the synthesis and secretion of cerebrospinal fluid in the central nervous system. Recent studies revealed multiciliated cells (MCCs) in the CP of the mouse [1–3]. Unlike ependymal cells that form multiple motile cilia to drive cerebrospinal fluid flow within the central nervous system after birth, MCCs in the CP epithelium arise during embryogenesis, display increased motility of their multiple cilia until birth, and experience a gradual regression in the motility during postnatal life [3–5]. Tumors of the CP comprise ~20% of brain tumors diagnosed in children under 1 year of age [6, 7]. Research aimed at understanding the origin and molecular characteristics of CP carcinoma (CPC) is essential for developing new therapies to improve clinical outcomes [8–12].

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MCCs on the epithelial lining of the brain ventricles, the airway, and reproductive tracts control fluid movement through the beating of multiple motile cilia on their apical surface. Multiciliogenesis is directed by a network of transcription factors that include two members of the Geminin family of coiled-coil containing nuclear proteins: Geminin Coiled-Coil Domain Containing (GMNC) and multi-ciliator differentiation and DNA synthesis associated cell cycle protein (MCIDAS) [13, 14]. GMNC and MCIDAS play sequential roles in the early steps of the MCC differentiation program that is triggered by NOTCH inhibition [15–20]. GMNC and MCIDAS activate the expression of downstream MCC factors, including forkhead box J1 (FOXJ1), v-myb avian myeloblastosis viral oncogene homolog (MYB), and cyclin O (CCNO), as well as TP53 family members TAp73 [21–28].

GMNC and MCIDAS both transcriptionally regulate multiciliogenesis in ependymal cells through the E2F4/S-DPI transcription factors [15, 16, 29]. In contrast, Geminin antagonizes the transcriptional functions of GMNC and MCIDAS. And Geminin and GMNC play antagonistic roles in the maintenance of the stem and ependymal cell populations in the adult neurogenic niche, respectively [30, 31]. CP epithelial cells are derived from neuroepithelial progenitors that express orthodenticle homeobox 2 (otx2) and Growth differentiation factor 7 (GDF7). As these progenitors exit the cell cycle to undergo multiciliogenesis and differentiation, TAp73 is activated in MCCs, while aquaporin 1 (AQP1), transthyretin (TTR), and cytokeratins are upregulated in differentiation, TAp73 is activated in MCCs, while aquaporin 1 progenitors exit the cell cycle to undergo multiciliogenesis and differentiate. The transcript lacking crucial 3'UTR, and cyto keratins are upregulated in ependymal cells [32]. Our previous work showed that, in contrast to all other MCCs, TAp73 was dispensable for multiciliogenesis in the CP, suggesting that its differentiation program may be distinct from other MCC types [26]. Therefore, further analysis of the molecular mechanisms governing multiciliogenesis in the CP, as well as the functional significance of these cilia, will be important to understand their role in the pathology of both ciliopathies and CP tumor development.

Examination of human CP tumors revealed abnormal NOTCH activity in a subset of tumors [33], and we demonstrated that sustained NOTCH1 expression in mice led to CP papilloma (CPP) that arose from monociliated progenitors in hindbrain roof plate [34, 35]. These progenitors proliferated in response to Sonic Hedgehog (SHH), but subsequently became quiescent after birth [34–36]. Here, we show that human CPC, and to a lesser extent CPP, display consistent defects in the GMNC-MCIDAS transcriptional program and amplification of NOTCH pathway components. Using two distinct murine models, we found that CPCs in mice exhibit multiciliation defects and a deficient GMNC program. In addition, persistent NOTCH and SHH signals are sufficient to drive aggressive tumors in mice that resemble human CPC. These tumors display singular primary cilia resulting from the repression of the GMNC-MCIDAS multiciliogenesis program by NOTCH. Biochemical or pharmacological disruption of the NOTCH complex restored multiciliation and suppressed tumor cell proliferation. Our findings indicate that the GMNC-MCIDAS transcriptional network is essential for MCC differentiation in the CP, and its activation can induce multiciliation and decrease CP tumor cell proliferation. These findings underscore the critical role of a compromised GMNC-MCIDAS multiciliogenesis program in CPC development and suggest that this could be exploited therapeutically to impair proliferation and promote tumor differentiation.

Results

CPs in humans exhibit reduced multiciliation and a deficient GMNC-MCIDAS program

Most CP tumors in humans, especially CPCs, consist of monociliated tumor cells and frequently display large-scale genomic alterations [34, 37–39]. Analysis of published data revealed recurrent chromosomal changes that affect loci encompassing multiciliogenesis regulators, including GMNC on chromosome 3, that is lost in all hypodiploid CPCs, MCIDAS, CCNO, microRNA 449 (MIR449), and CDC208, that are all located within the same locus of chromosome 5, and MYB on chromosome 6, that is lost in many CPCs (Fig. 1A). Conversely, N-acetyl galactosamine-type O-glycosylation enzyme GALNT11, a positive regulator of NOTCH signaling on chromosome 7, is gained in >80% CP tumors (Fig. 1A) [37–40]. In agreement, among 11 cases of human CPCs examined, most displayed significantly reduced or complete loss of GMNC expression, and GMNC expression was heterogeneous and only detected in a subpopulation of tumor cells (Fig. 1B, C). Decreased FOXJ1 expression was also observed in the majority of samples (Fig. 1B, C). A similar trend of reduced GMNC and FOXJ1 expression was observed in 31 human CPPs (Fig. 1B, C). Consistent with the requirement for GMNC and FOXJ1 in MCC differentiation, analysis of cilia marker ADP-ribosylation factor-like 13b (ARL13B) in six CPCs found that all were monociliated, while 11 of 17 CPPs analyzed were monociliated (Fig. 1B, D).

Accordingly, RT-qPCR analysis revealed low levels of GMNC and MCIDAS expression in most human CP tumors compared to normal tissues, while FOXJ1 expression in CPCs was significantly lower than CPPs (Fig. 1E). In contrast, TAp73 expression in human CPC and CPP varied from significantly reduced to normal levels in similar proportions (Supplementary Fig. S1A). Moreover, analysis of a published dataset revealed differential expression of genes involved in ciliogenesis in human CP tumors, contributing to significant enrichment of the pathway (Supplementary Fig. S1B, C) [37]. Thus, CPCs in humans are characterized by multiciliation defects and deficiencies in the GMNC-MCIDAS program, as well as recurrent amplification of NOTCH regulators.

Gmnc-Mcidas signaling is essential for generating multiciliated epithelia in the CP

These results suggest that suppressing the MCC fate program controlled by GMNC and MCIDAS was a key step in the genesis of CP tumors. While GMNC was implicated in the formation of MCCs in the CP [15], a detailed examination of its role in the CP has not been carried out. The Gmnc conditional allele (Gmnclox/flox) has two loxP sites located in introns 3 and 5 that allow Cre-mediated deletion of exons 4/5 to generate the null allele (Gmnc−/−) [15]. Using electron microscopy and immunostaining, we compared wild type CP to animals with a conditional deletion of Gmnc in the roof plate/CP by the Lmx1a-Cre transgene [41]. We found that both ependymal cells and the CP epithelium of Gmnc-/- animals were comprised solely of monociliated cells, compared to wild type controls that exhibited multiple basal bodies and multiciliation (Fig. 2A, B; Supplementary Figs. S2A, B, S3A). RT-qPCR using primers from exons 4/5 revealed significantly reduced Gmnc levels in the CP from Lmx1a-CreGmnclox/fllox mice at postnatal (P) day 7 (P7), consistent with efficient Gmnc disruption (Fig. 2C). Analysis of Ki-67 expression showed that both Gmnc−/− and wildtype CP epithelial cells became postmitotic and the expression of epithelial markers cytokeratins, TTR, and otx2 was comparable between Gmnc−/− and wild type CP, though Aqp1 expression was significantly increased in Gmnc−/− animals (Fig. 2C; Supplementary Fig. S3B–F).

Gmnc mRNA was detected in wild type CP epithelial cells adjoining the roof plate at embryonic (E) day 13.5 (E13.5), and Gmnc transcripts persisted in the epithelial cells of Gmnc−/− CP (Fig. 2D). RT-qPCR with primers from exons 2/3 showed increased Gmnc levels, and further sequencing revealed a mutant transcript with exon 3 spliced to exon 6 (designated as Gmnc1344S) (Fig. 2C; Supplementary Fig. S4A). The altered splicing causes a frame shift and stop codon after a few amino acids, generating a truncated Gmnc transcript lacking crucial functional domains. The expression of Gmnc targets Foxj1 and TAp73 in the CP and ependyma was markedly reduced in Gmnc−/− mice, whereas Gmnc overexpression stimulated TAp73

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expression (Fig. 2C, D; Supplementary Figs. S4B, D, S5A, C, S14, Supplementary Table 1). Together, these data establish that Gmnc is essential for MCC differentiation and the expression of TAp73 and Foxj1 in the CP epithelium.

We next examined critical components of the MCC transcriptional cascade, including Mcidas, Myb, and Ccno. All 3 genes were transiently upregulated in a subpopulation of epithelial cells next to the roof plate during development and their expression was lost in Gmnc−/− mice (Fig. 2D; Supplementary Fig. S5B), indicating that GMNC activates the MCIDAS-dependent program in the CP, as has been shown in other MCC-containing tissues. Examination of CP epithelial cells in Mcidas−/− mice revealed only solitary primary cilia, and OTX2 and AQP1 expression were similar to that of the wild type CP (Fig. 2E; Supplementary Fig. S6A, B). The expression of Gmnc, Foxj1, and TAp73 remained unaltered by Mcidas loss, although Mcidas overexpression stimulated TAp73 and Ccno expression (Supplementary Figs. S4C, D, S6C, D, S14, Supplementary Table 1). Taken together, these results indicate that MCIDAS plays a critical role in multiciliogenesis downstream of GMNC in the CP.

Gmnc loss mediates cilia defects in CPCs with deficient Rb1/Trp53 signaling

CPC frequently occurs in Li-Fraumeni syndrome patients and somatic TP53 mutations in sporadic CPC predict poor outcome [37, 42]. In mice, Trp53 deletion combined with Rb1 loss or Myc overexpression leads to CPC with characteristics of their human counterparts [8–10, 42–44]. We crossed Lmx1a-Cre mice that express Cre in the roof plate/CP [41], with a mouse strain carrying conditional alleles of Trp53 and Rb1 (p53fl/fl;Rbfl/fl) to cause their deletion in CPC progenitors [45]. All Lmx1a-Cre; p53fl/fl;Rbfl/fl (Lcre;p53cko;Rbcko) mice developed CPC, characterized by higher numbers of Ki-67+ proliferative cells (Fig. 3A). As early as 9 weeks after birth, a small population of Gmnc-negative cells was detected in the CP of Lcre;p53cko;Rbcko animals, whereas OTX2+ monociliated cells were present among MCCs in the CP at 11 weeks of age (Fig. 3B, C). Tumor cells in these mice were monociliated and exhibited significantly reduced Gmnc and Foxj1 levels (Fig. 3B–D; Supplementary Fig. S7A; Supplementary Table 1). Thus, Rb1/Trp53-deficient murine CPC recapitulates the multiciliation defects and GMNC program deficiencies in CPC in humans.
The loss of Gmnc expression at early stages of tumorigenesis suggested that this may promote the reduced multiciliation observed in Rb1/Trp53-deficient CPC. To address this, we interbred Lcre;p53cko;Rbcko mice with Gmnc<sub>fl</sub>ox/− animals. The resulting Lmx1a-Cre;Lcre;p53cko;Rbcko;Gmnc<sub>fl</sub>ox/− (Lcre;p53cko;Rbcko;Gmnc<sub>cko</sub>) mice succumbed to CPCs that expressed OTX2 and showed proliferation levels similar to that of Lcre;p53cko;Rbcko mice (Fig. 3A; Supplementary Fig. S7B). The expression of Ttr, Aqp1, Gdf7, and Foxj1 was significantly reduced, whereas TAp73 expression was more variable in CPC in these mice (Fig. 3D; Supplementary
signal were sufficient to drive more aggressive CPC-like tumors, we bred a mouse strain carrying a \(\text{Gmnc}^{\Delta 4-5}\) conditional allele to detectable in the CP of the \(Lrcre^{\text{Gmnc}^{\Delta 4-5}}\) mouse, but its presence was dramatically reduced in tumor Fig. 5A, E; Supplementary Fig. S10C, D). Consistently, tumor cell proliferation was markedly reduced, and the expression of Foxj1 was significantly increased by IMR-1 (Fig. 5D, E; Supplementary Fig. S10B). After a 7-day in vivo IMR-1 treatment from day E10.5, multiciliated tumor cells were detected in Lcre;NICD1 and Lre; \(\text{Ptc}^{\text{Chox}}\);NICD1 animals at day E17.5 and day P7 (Fig. 6A, B). This was accompanied by a significant decrease in tumor cell proliferation, and a reduction of total tumor cell numbers by several folds at day P7 (Fig. 6C, D). Moreover, the expression of SOX2 in the ventricular zone, PAX6, and Atoh1 in progenitors derived from rhombic lip was comparable between wild type animals treated with IMR-1 or vehicle (Supplementary Fig. S10C, D). Together, these results demonstrate that aberrant NOTCH signaling impairs MCC differentiation in the CP that can be rescued by NOTCH inhibition, leading to reduced tumor growth.

\section*{Gmnc suppression by NOTCH mediates defective multiciliation in CP tumors}

To understand the mechanisms of MCC regulation in tumor cells, we integrated RT-qPCR, RNAseq, and spatio-temporal gene expression data. Results from these assays consistently showed that both Foxj1 and Micsad were expressed in tumor cells at lower levels than observed in wild type CP epithelium (Fig. 7A; Supplementary Fig. S11A-D) [34]. As this suggested that upstream regulators of the MCC program were impaired, we examined Gmnc expression. Although Gmnc exhibited ubiquitous expression in the CP epithelium, we consistently observed decreased levels of Gmnc and its downstream target TAp73 in CP tumors (Fig. 7A, B; Supplementary Fig. S11C, D). This was accompanied by a transient increase in the expression of Gmnn, a gene that is normally associated with proliferation and was shown to antagonize Gmnc transcriptional functions (Fig. 7B) [15, 30]. These results demonstrate that the Gmnc-MCIDAS program is profoundly repressed in NOTCH-driven CP tumors, and this can be modulated using NOTCH pathway inhibitors.
To understand the role of the GMNC-MCIDAS program in defective multiciliation of CP tumors, myc-tagged GMNC or MCIDAS was expressed in tumor cells from Lcre;NICD1 mice using viral vectors. Enforced expression of Gmnc or Mcidas led to the formation of multiple cilia and reduced proliferation in infected tumor cells within 72 h (Fig. 7C, D; Supplementary Fig. S12A, B), phenocopying NOTCH inhibition with IMR-1 that significantly increased Gmnc levels in tumor cells (Fig. 7E). We subsequently eliminated Gmnc by crossing Gmncfllox/− and Lcre;NICD1 animals. Tumor cells from Lcre;NICD1;Gmncfllox/− mice became resistant to multiciliation, Foxj1 activation, and decreased proliferation induced by IMR-1/IMR-1A (Fig. 7F–H; Supplementary Fig. S12C, D). Conversely, overexpression of Gmnc increased Foxj1 expression in tumor cells (Supplementary Fig. S12E). Therefore, these results indicate that monociliation in tumor cells is maintained through NOTCH suppression of GMNC-MCIDAS signaling and suggest that GMNC loss prevents the rescue of multiciliation defects by NOTCH inhibition.
Similar to Rb1/Trp53-deficient CPC, despite the suppression of the GMNC-MCIDAS program by NOTCH, combined loss of Gmnc and Patched1 failed to induce CPC in Lcre;Ptch1tm1Hahn;Gmncflox/-; mice (Supplementary Fig. S13; Supplementary Table 1), suggesting that loss of GMNC-driven multiciliation in the CP is insufficient to replace NOTCH or Rb1/Trp53 deletion in CPC. Together, these data indicate that GMNC-MCIDAS program deficiencies critically mediate cilia defects in CPC to modulate tumor growth.

**DISCUSSION**

CPC clinical outcomes remain dismal, leaving patients vulnerable to devastating consequences [2, 3]. The gross genomic alterations in CP tumors have made the identification of driving events and actionable targets difficult [43, 44]. The GMNC-MCIDAS program promotes multiciliogenesis in different tissues, is required for MCC generation in mice, and mutations in both GMNC and MCIDAS have been identified in human ciliopathies [13, 14]. The observation that there is consistent disruption of multiciliogenesis program and prevalence of solitary cilia in CPC indicates that CPC has characteristics of a ciliopathy and that therapeutic strategies aimed at restoring multiciliogenesis may suppress CP tumors.

Our findings revealed the interaction of the multiciliogenesis program, NOTCH, and SHH pathways during CP differentiation and tumorigenesis. NOTCH suppressed multiciliation of roof plate progenitors, thereby preserving cilia-based signaling activated by SHH from postmitotic MCCs in CP epithelium [33]. Conversely, SHH signaling enhanced Hes1 and Hes5 expression in the roof plate in Lcre;Ptch1tm1Hahn mice and NOTCH-driven CPC tumors. The expanded upper roof plate in Lcre;Ptch1tm1Hahn;Ncid1 mice is consistent with the developmental origin and cilia defect of CPC being driven by NOTCH and SHH signaling. These animals represent an ideal therapeutic model for congenital or infantile CPC, a rare condition associated with high morbidity and mortality [50, 51]. Indeed, NOTCH inhibition by IMR-1 rescued the cilia defect by inducing multiciliated tumor cells, whereas SHH pathway inhibitors suppressed tumor cell proliferation (Fig. 8) [34]. Thus, further study of the interactions between the SHH and NOTCH pathways in CP tumors is warranted to determine the therapeutic potential of activators of multiciliation and cilia-dependent signaling [52–56].

While the differentiation of MCCs requires NOTCH inhibition, it is unclear precisely how NOTCH impacts MCC fate during tumorigenesis [57–61]. Our data shows for the first time that NOTCH suppresses the expression of Gmnc and Mcidas to impair multiciliation during tumorigenesis. Gmnc is required for MCC differentiation following NOTCH inhibition, indicating that GMNC-MCIDAS signaling is required downstream of NOTCH regulation and represents a potent anti-tumor mechanism in CP tumors (Fig. 8).

Consistent with previous studies, we found that the GMNC-MCIDAS program was required for multiciliation in the CP epithelium. As progenitor cells exit the cell cycle to undergo multiciliogenesis, the expression of Gmnc, Mcidas, Foxj1, and TAp73 was upregulated, as has been observed in other multiciliated tissues [26]. Ectopic expression of either GMNC and MCIDAS stimulated Foxj1 and TAp73 expression, whereas GMNC loss prevented the activation of Mcidas, TAp73, and Foxj1, in contrast to Mcidas-deficient MCCs that showed expression of both Foxj1 and TAp73 [14, 25]. Although both TAp73 and Foxj1 are sensitive to GMNC status, loss of TAp73 failed to affect Foxj1 expression in the CP, as it does in other MCCs, indicating that TAp73 is not integrated into the Gmnc-Foxj1 axis in the CP [26]. Consistent with this, TAp73 expression varied greatly in Rb1/Trp53-deficient CPC in mice, as well as CP tumors in humans. These results highlight the need to further analyze the similarities and differences between different MCC types.

Overall, this study shows that the GMNC-MCIDAS program is required for MCC differentiation in the CP. The impairment of the program by oncogenic signals including Rb1/Trp53 defects or NOTCH activation prevents multiciliation and facilitates proliferation of CP tumor cells (Fig. 8). Therefore, activation of multiciliogenesis may serve as a potential therapeutic strategy in a subset of CP tumors. As the early events leading to the activation of the GMNC-MCIDAS program remain poorly characterized, a detailed understanding of its regulation and functions will be critical for developing strategies to target this pathway for the treatment of CPC.

**MATERIALS AND METHODS**

**Animals**

Gmnc(Rosa26Sor+tm1.Notch1Dam/J; Gmncflox/-); mice, B6.129P2-Ptch1tm1Hahn/J (Ptch1flox/flox) mice, B6.129P2-Trap2tm1mbr/J (Mcidasflox/flox) mice, B6.129P2-Rbp4tm1Jrb/J (Rbp4flox/flox) mice, and C57BL/6 mice (all from Jackson Laboratory, Bar Harbor, ME, USA), Tg(Lmx1a-cre);K1my (Lmx1a-Cre) mice, Gmncflox/-; Mcidasflox/-; mice were maintained in compliance with national regulatory standards. Mcidas mutant mice were housed at the Biological Resource Center of the Agency for Science, Technology and Research (A*STAR) of Singapore, and experiments performed with these animals followed guidelines stipulated by the Singapore National Advisory Committee on Laboratory Animal Research. All experimental procedures at the Institute for Research in Biomedicine were conducted following European and National Regulation for the Protection of Vertebrate Animals used for experimental and other scientific purposes (directive 86/609), internationally established 3R principles, and guidelines established by the United Kingdom Coordinating Committee on Cancer Research.

The animal experiments were not randomized, and both male and female animals were used for experiments at different time points. For analysis of the Gmnc-Mcidas program in MCC differentiation in the CP,
Fig. 4 Aberrant NOTCH and SHH signaling drive CPC in mice. A Wild type, Lcre;NICD1, Lcre;Ptch<sup>cko</sup>, and Lcre;Ptch<sup>cko</sup>;NICD1 animals are shown at day E14.5. Notice the cranium defects resulting from enlarged and folded roof plate in the midbrain-hindbrain region of Lcre;Ptch<sup>cko</sup> and Lcre;Ptch<sup>cko</sup>;NICD1 animals (white arrowheads). H&E staining and Ki-67 expression are shown of roof plate (upper roof plate marked by red lines) and the CP (black arrows) in the hindbrain in wild type and Lcre;Ptch<sup>cko</sup> animals, and CPP and abnormal CP growth (black arrowheads) in Lcre;NICD1 and Lcre;Ptch<sup>cko</sup>;NICD1 animals, respectively. Enlarged roof plate disrupts the cranium in Lcre;Ptch<sup>cko</sup> and Lcre;Ptch<sup>cko</sup>;NICD1 animals (red arrows). The upper roof plate is shown in higher magnification in the right (Lcre;Ptch<sup>cko</sup>;NICD1 animal) and lower (wild type, Lcre;NICD1, Lcre;Ptch<sup>cko</sup>, and Lcre;Ptch<sup>cko</sup>;NICD1 animals) panels. Scale bars, 100 µm. Quantification of Ki-67 expression in the upper roof plate and CP in the hindbrain is shown (wild type mice: n = 11; Lcre;NICD1 mice: n = 4; Lcre;Ptch<sup>cko</sup> mice: n = 3; Lcre;Ptch<sup>cko</sup>;NICD1 mice: n = 7 for upper roof plate, n = 8 for the CP; mean ± s.e.m., one-way ANOVA, ***p < 0.001; ****p < 0.0001). Data are representative of at least three independent experiments. B Representative results of immunohistochemical staining for OTX2, and AQP1 are shown in the upper roof plate (marked by dotted lines) and the CP (arrows) in the hindbrain at day E14.5 in wild type and Lcre;Ptch<sup>cko</sup> animals, and CPP and abnormal CP growth (black arrowheads) in Lcre;NICD1 and Lcre;Ptch<sup>cko</sup>;NICD1 animals, respectively. Residual AQP1-expressing epithelial cells (red arrowhead) are mixed with tumor cells in Lcre;NICD1 animals. Scale bar, 50 µm. Images represent at least three independent experiments.
animals analyzed included: Gmnc^{−/−} Mcidas^{−/−}, and wild type animals (n = 3 for each at genotype at each time point). The investigators were blinded to group allocation during experiments and assessment of tumor development in wild type (n = 16), Lmx1a-Cre;Ptch^{lox/lox};Rb^{lox/lox};Gmnc^{−/−} (n = 17), and Lmx1a-Cre;Ptch^{lox/lox};Rb^{lox/lox};Gmnc^{−/−};Lcre;NICD1 (n = 36) mice. For NOTCH-driven CP tumors, animals analyzed used included: wild type (n = 11), Lmx1a-Cre;NICD1 (n = 4), Lmx1a-Cre;Ptch^{lox/lox};Lcre;NICD1 (n = 3), and Lmx1a-Cre;Ptch^{lox/lox};NICD1 (n = 8) mice. For analysis of the role of Gmnc in NOTCH-driven CP tumors, 3 animals were used for wild type, Lmx1a-Cre;Ptch^{lox/lox};Gmnc^{−/−}, Lmx1a-Cre;Ptch^{lox/lox};Gmnc^{−/−};Lcre;NICD1 (vehicle n = 14; IMR-1: n = 13), and Lmx1a-Cre;Ptch^{lox/lox};NICD1 (n = 5 for vehicle or IMR-1, respectively).
Human samples
CP specimens were procured with informed consent from patients following the requirements by institutional review boards at Shanghai East Hospital, Sanford Burnham Prebys Medical Discovery Institute, and University Medical Center Hamburg-Eppendorf. All CP specimens from Boston Children’s Hospital were obtained under an approved institutional review board protocol. 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/o_ce-clinical-research-and-bioethics-policy). Diagnoses of human CP specimens from Boston...
Children's Hospital were reviewed by two neuropathologists (HGWL, S. Santagata) using standard WHO criteria [62].

Cell culture

Multiple sets of tissue specimens were collected from animals of appropriate genotype and maintained in culture. Gender information is not available for animals collected at day P7. Primary CP tumor cells were cultured as described previously [34]. Dissected specimens were dissociated and digested at 37 °C for 20 min with pronase (2 mg/ml, 537088, EMD Millipore, Darmstadt, Germany) or collagenase type IV (2 mg/ml, 573088, EMD Millipore, Darmstadt, Germany), depending on specimen type. Dissociated tumor cells were centrifuged at 100 g for 2 min at 4 °C. Cells were resuspended and cultured in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 Ham's Liquid Media (DMEM:F12, SH30261; HyClone Laboratories, Waltham, MA, USA) supplemented with 20% fetal bovine serum (FBS, R&D Systems, Inc., Minneapolis, MN, USA) and 100 μg/ml penicillin/streptomycin (Thermo Fischer Scientific, Waltham, MA, USA) or 10% FBS (R&D Systems, Inc.) and 100 μg/ml penicillin/streptomycin (Thermo Fischer Scientific). Mouse inner medullary collecting duct cells (mIMCD3, ATCC, CRL-2123) were cultured in DMEM:F12 Medium (HyClone Laboratories) supplemented with 10% FBS (R&D Systems, Inc.) and 100 μg/ml penicillin/streptomycin (Thermo Fischer Scientific). These cells were maintained in a humidified atmosphere with 5% CO2 at 37 °C in a cell culture incubator.

Analyses of gene expression, cell proliferation, and signal transduction were performed using CP tumor cells from Lmx1a-Cre;NICD1 (n = 13), Lmx1a-Cre;NICD1 GMTGC−/−, GMTGC−/− mice (n = 3). Results from these studies confirmed their identity [Figs. 3E, 5B–H; Supplementary Figs. 5A–D, 5C, 6A, 7G–I, 8]; see also Figs. 5B, 6G, 7A, 8A, and 9A). The percentage of MCCs was determined by calculating the number of MCCs per 100 tumor cells or infected cells. The percentage of Ki-67+ cells was determined by calculating the number of Ki-67+ cells per 100 cells in each sample for each genotype or each treatment. For proliferation analysis of cultured cells, Ki-67 expression in three distinct regions of each sample. The percentage of Ki-67+ cells per 100 cells in each sample for each genotype or each treatment. For proliferation analysis of cultured cells, Ki-67 expression in three distinct regions of each sample. The percentage of Ki-67+ cells was determined by calculating the number of Ki-67+ cells per 100 tumor cells or infected cells in each sample for each treatment.

For analysis of multicellular tumor cells, primary cilia of 100–150 tumor cells or infected cells were assessed by analyzing ARL13B or γ-tubulin expression in three distinct regions. The percentage of MCCs was determined by calculating the number of MCCs per 100 tumor cells or infected cells of each sample for each treatment.

Immunoblotting

Immunoblotting was carried out as described previously [34]. Primary antibodies used included: mouse monoclonal anti-β-Actin (1:1000, clone AC-15, Sigma-Aldrich), and rabbit anti-Tap73 (1:1000, ab40658, abcam), and mouse monoclonal anti-FLAG (1:1000, clone M2, F3165, Sigma-Aldrich).

RT-qPCR, in situ hybridization and RNAscope

Multiple sets of tissue specimens were collected from animals. Gender information is not available for animals collected at days P0 and P7. Total RNA was extracted from tumor samples using Trizol (Thermo Fischer Scientific) and RNA Clean & Concentrator kits (Zymo Research, Irvine, CA, USA). Total RNA samples from normal human tissues were purchased (BioChain Institute, Inc., Newark, CA, USA). cDNA was synthesized using GoScript Reverse Transcription System (Promega, Madison, WI, USA). All reactions were run on a QuantaStp3 3 Real-Time PCR System (Thermo Fisher Scientific). Gene-specific primers and probes were used (Gdf7, Mm. PRT.SB.121004445; Gmnc, Hs.PT.SB.15454001; MCDAS, Hs.PT.SB.22676071; FKIRPB, Hs.PT.SB.40371261; Integrated DNA Technologies, Inc., Coralville, IA, USA) (Supplementary Table 2) [34]. Transcript levels were determined as the number of transcripts of genes of interest relative to those of Actb (mouse) or GAPDH (human) and normalized to the mean value of control samples. The results for each set of specimens were obtained by averaging

Fig. 6 NOTCH inhibition restores multicellularity in CP tumors. Representative images of immunofluorescent staining for ARL13B (A, yellow; B, red) are shown in tumor cells at day E17.5 (A) and tumor cells isolated at day P7 (B) from Lcre;NICD1 (A, B, red) and Lcre;Petch3′′;NICD1 (A) animals treated with vehicle or IMR-1 from day E10.5 to day E16.5. Boxed region of ciliated cells is magnified in lower panel (A). DAPI staining (A, cyan; B, blue) labels nuclei. Scale bars, 5 μm (A), 10 μm (B). Results were obtained from at least three independent experiments. C Quantification total tumor cell numbers isolated at day P7 is shown in Lcre;NICD1 animals treated as described in A and B (n = 5 animals per treatment; mean ± s.e.m., two-tailed unpaired t-test, ***P < 0.001). D The expression of Ki-67 (n = 5 in NICD1; GFP+ tumor cells at day E17.5 is shown in Lcre; NICD1 animals treated with vehicle or IMR-1 from day E10.5 to day E16.5. DAPI staining (blue) labels nuclei. Scale bar, 20 μm. Quantification of Ki-67 expression in tumor cells is shown (right panel: n = 9 animals for vehicle, n = 8 animals for IMR-1; mean ± s.e.m., two-tailed unpaired t-test, **P < 0.01). Data are representative of three independent experiments.
transcript levels of technical triplicates and used for subsequent analyses. Exclusion was applied when one of the triplicates was a significant outlier, and the assay was repeated in independent experiments to validate the exclusion. For analysis of GMNC, MCIDAS, and FOXJ1 expression, human samples used included 10 CPPs, 8 CPCs, and 1 for brain, trachea, lung, testis, and epididymis. For analysis of Gmnc-deficient CP samples, animals included: 11 Lmx1a-Cre;Gmnc<sup>floxed</sup>− and wild type animals, respectively. For CP tumor analysis, samples used included: wild type CP: n = 10; CPC: n = 11 from Lmx1a-Cre; p53<sup>floxed</sup>/p53<sup>floxed</sup>;Rb<sup>floxed</sup>/Rb<sup>floxed</sup> mice, n = 10 for Lmx1a-Cre; p53<sup>floxed</sup>/p53<sup>floxed</sup>;Rb<sup>floxed</sup>/Rb<sup>floxed</sup>;Gmnc<sup>floxed</sup>− animals. For NOTCH-driven CP tumors, animals examined included: wild type, Lmx1a-Cre;NICD1 (n = 3 for each at genotype at each time point). For gene expression analysis of infected Q. Li et al.
**Fig. 7** Gmnc suppression by NOTCH mediates multici llation defects in CP development and tumorigenesis. A Median FKPM (fragments per kilobase of exon per million reads mapped) values of genes in NOTCH-driven CP tumors and wild type CPs (n = 3 specimens per time point, mean ± s.e.m., two-tailed unpaired t-test, *P < 0.05; **P < 0.01). B RT-qPCR analysis of NOTCH-driven CPP and wild type CP (n = 3 animals per time point, mean ± s.e.m., two-tailed unpaired t-test, ***P < 0.001, ****P < 0.0001). Three independent experiments were conducted. C The expression of ARL13B (red) is shown in tumor cells infected with viruses expressing GMNC-myc, MCIDAS-myc, or control only. GMNC-myc (green), or MCIDAS-myc (green) labels infected cells. DAPI staining (blue) labels nuclei. Scale bar, 20 µm. Quantification of the percentage of MCCs in infected cells is shown on the right (n = 4 per treatment, mean ± s.e.m., one-way ANOVA, *P < 0.05). Results were obtained from three independent experiments. D The expression of Ki-67 (red) is shown in tumor cells from Lcre;NICD1 mouse infected with viruses expressing GMNC-myc. GMNC-myc (green) labels infected cells. DAPI staining (blue) labels nuclei. Scale bar, 20 µm. Quantification of Ki-67 expression in tumor cells from Lcre;NICD1 mouse infected with viruses expressing GMNC-myc or control vectors is shown in the lower panel (n = 6 per treatment, mean ± s.e.m., two-tailed unpaired t-test, ***P < 0.001). Data represent at least three independent experiments. E Representative images of Gmnc expression (green) by RNAscope are shown in tumor cells treated with vehicle or IMR-1. DAPI staining (blue) labels nuclei. Scale bar, 20 µm. Quantification of Gmnc transcript is shown (n = 7 per treatment, mean ± s.e.m., two-tailed unpaired t-test, ***P < 0.001). Three independent experiments were conducted. F The expression of ARL13B (red) is shown in Gmnc-deficient tumor cells treated with vehicle or IMR-1/IMR-1A. GFP (green) labels tumor cells. DAPI staining (blue) labels nuclei. Scale bar, 20 µm. Data represent five independent experiments. G RT-qPCR analysis of Foxj1 expression in tumor cells treated with vehicle or IMR-1 (tumors from Lcre;NICD1 mice: n = 6 per treatment; tumors from Lcre;NICD1;Gmnclox-mice: n = 4 per treatment, mean ± s.e.m., paired t-test, *P < 0.05, NS, not significant). Results were obtained from three independent experiments. H Quantification of Ki-67 expression is shown in Gmnc-deficient tumor cells treated with vehicle or IMR-1 (n = 6 per treatment, mean ± s.e.m., two-tailed unpaired t-test, NS not significant). Three independent experiments were conducted.

**Fig. 8** Schematic diagram of GMNC-MCIDAS program in CP development and tumorigenesis. A Combined activation of NOTCH and SHH signaling, or loss of Rb1/Trp53 tumor suppressors drives CPC development. B GMNC-MCIDAS program mediates multici llation in CP epithelium, and is repressed by NOTCH signaling in roof plate progenitors, whereas NOTCH inhibitor IMR-1 promotes GMNC-dependent multici llation and suppresses tumor growth. GMNC-MCIDAS program suppression in Trp53-deficient CPC maintains multici lated tumor cells.

used included: 31 CPPs from 31 individuals; 11 CPs from 11 individuals. GEMC1 and FOXJ1 expression was assessed in five distinct tissue regions: the percentage of GEMC1- or FOXJ1-expressing cells was calculated by averaging the numbers of GEMC1+ or FOXJ1+ cells per 100 cells in five distinct tissue regions of each specimen. For RNAscope in cultured or infected tumor cells, 7 samples were used for each treatment. mRNA transcript copy number was assessed by counting the number of positive fluorescent spots in 50–100 tumor cells or infected cells in three distinct regions. The transcript levels were determined by averaging the transcript copy numbers of all cells for each treatment.

**Electronic microscopy and image acquisition**

Transmission electron microscopy was performed as described previously [34]. The investigator was blinded to group allocation. A whole-mount bright field was obtained using a Nikon SMZ1000 Stereomicroscope. Light and fluorescent microscopic images were obtained by a Nikon Eclipse 90i microscope system, a Nikon confocal microscope system A1+ (Nikon Instruments, Melville, NY, USA), and a ZEISS LSM 980 with Airyscan 2 confocal microscope (Carl Zeiss Microscopy, LLC, White Plains, NY, USA).

**Statistical analysis and reproducibility**

Multiple specimens were collected from independent samples or animals for each treatment or genotype. Pilot studies were conducted, and results from these studies were used to determine the choice of sample size for the experiment. A group size of n = 10 (5 experimental, 5 control) will provide 90% power to detect a 22% change in assay results. No randomization was used to determine how samples were allocated to experimental groups. Both male and female animals were used for experiments. Experiments were repeated with similar results to eliminate the effects of gender and age on experimental findings. Information on experiment replication is provided in legends for figures and supplemental figures. Statistical analyses were performed with GraphPad Prism 9.0 (GraphPad Software Inc., La Jolla, CA, USA). All pooled data were expressed as the mean ± standard error of the mean (SEM). Variation within each group of data was examined based on the differences between each data point and the mean of the group. The Kolmogorov–Smirnov test was used to test the normal distribution of the data. Differences between two groups were compared using paired t-test or unpaired two-tailed t-test. Differences between multiple groups were analyzed with ANOVA followed by Tukey’s multiple comparisons test. Results were considered significant at *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

**Accession numbers**

Published data sets of human CP tumors (GSE14098, GSE60886) were downloaded from the GEO database. Hierarchical clustering was performed using Genesis (http://genome.tugraz.at/genesisclient/genesisclient_description.shtml). Pathway analysis using the GeneGoMetaCore Analytical Suite (http://genego.com; GeneGo) was used to score and rank pathways enriched in data sets by the proportion of pathway-associated genes with significant expression values. RNA-seq data (BioProject ID, PRJNA282889) were analyzed.
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**AUTHOR CONTRIBUTIONS**

QL, ZH, ZL, and HZ conceived and designed the study; SR, ZL, THS, and HZ performed development of methodology and writing, review, editing, and revision of the manuscript; QL, ZH, NS, BT, RMF, MKL, HGWL, UA, TDP, JQV, and YZ provided acquisition, analysis and interpretation of data, and statistical analysis; TZ, AA, YH, PC, HL, SR, JW, RJW, KS, LW, US, and ZL provided technical and material support. All authors read and approved the final paper.

**COMPETING INTERESTS**

The authors declare no competing interests.

**ETHICS APPROVAL AND CONSENT TO PARTICIPATE**

Tissue samples were obtained with informed consent from patients and approval by institutional review boards at Shanghai East Hospital, Sanford Burnham Prebys Medical Discovery Institute, and University Medical Center Hamburg-Eppendorf. All tissue specimens from Boston Children’s Hospital were obtained under an approved institutional review board protocol. The study was performed in accordance with the Declaration of Helsinki.

**ADDITIONAL INFORMATION**

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